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The Editor comments

WHAT'S IN A NAME?

In recent years there has been increasing demand from various quarters that nonproprietary or so-called "generic names" coined for new drugs be short, readily pronounced, and easily committed to memory. However, those responsible for selecting these names have attempted to point out at least two other important considerations; namely, that such names should, if possible, be chemically descriptive and not subject to confusion with existing names. These additional restrictions are most important, but often increase the length and complexity of the resulting name. Those individuals not experienced in the development and assignment of nonproprietary nomenclature generally do not appreciate the difficulty of deriving a suitable name which will reasonably meet the above tests.

In an unusually candid and forthright statement, Dr. L. S. Goodman recently told the Kefauver Committee:

"This nomenclature problem is sticky. I once believed that drug companies, on occasion, deliberately and gleefully proposed generic names so polysyllabic and so unpronounceable that even a simon-pure pharmacologist would gladly settle for a short, euphonious trade name in his teaching of medical students and in his lectures to practicing physicians. Upon becoming a member of the AMA Council on Drugs, I learned that I was wrong."

The charge has further been made from a number of poorly-informed sources that the nonproprietary names presently being assigned do not, in fact, completely describe the chemistry of the drug. This certainly is true, but nevertheless does not eliminate their usefulness. The nonproprietary name is no more a substitute for the chemical name than is a police description of a fugitive a substitute for a set of fingerprints. Like the police description, however, the nonproprietary name is useful because it often will rule out the great majority of other possibilities even though it does not completely identify a single entity. An example is the name *tolazamide* which was recently suggested for the drug chemically described as 1-(*p*-tolylsulfonyl)-3-(hexahydro-1-azepinyl)urea. The proposed name is quite informative since it indicates that the compound referred to contains: (*a*) a tolyl group, (*b*) at least two nitrogen atoms in a direct linkage, and (*c*) some type of amide moiety. No more than one organic compound in a thousand would be characterized by all three of these distinctive features, and numerous possibilities are thereby eliminated.

Therefore, we feel it should be emphasized that while the nonproprietary name does not usually tell what the drug *is*, almost equally important, it often tells what the drug *is not*.

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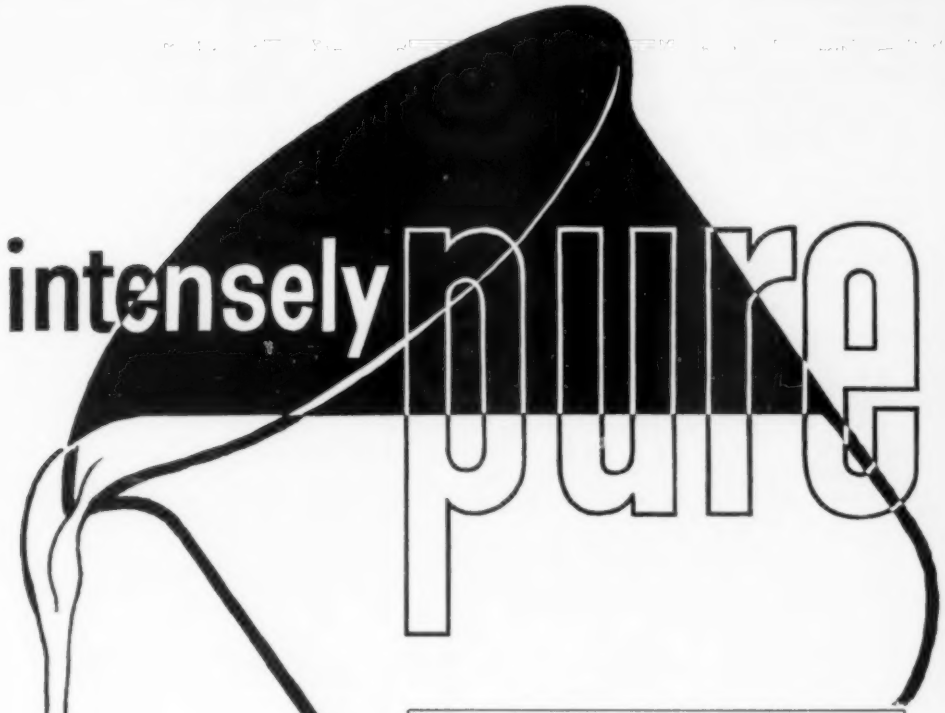

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Review Article

Absorption, Testing, and Clinical Evaluation of Oral Prolonged-Action Drugs

By JACK LAZARUS and JACK COOPER

THE POSSIBILITY of favorably modifying by purely pharmaceutical means the therapeutic response to drugs administered by mouth provided a powerful stimulus to research workers in pharmacy and medicine. Here was an "open sesame" of scientific opportunity further triggered by vistas of economic rewards. The speed with which numerous pharmaceutical laboratories jumped into the race resulted in a plethora of ideas and products many of which should have remained unborn. Nevertheless, from out of this exciting ferment there has already come new formulations, new techniques, new tests, and new chapters in standard pharmacy reference books.

Almost a decade has elapsed since the first long-acting dosage form for oral use was introduced. The favorable reception accorded to the prolonged-action dosage forms by the medical profession is reflected in the annual total volume registered by these preparations many of which are available only upon prescription. According to Maurina (1), sales of long-acting products in the United States exceeded $87,000,000 in 1959, a volume representing 4 per cent of the total pharmaceutical market. The increasing number of prolonged-action preparations making their appearance on the market evidences the growing interest of the pharmaceutical industry in these forms. Market research estimates indicate a sales volume exceeding $100 million for 1961. Three firms dominated the field in 1959, controlling 73.4 per cent of the market in terms of total sales with the balance distributed among forty-eight manufacturers. Approximately 180 different products are available in oral prolonged-action form, with 20 of these from large pharmaceutical companies and many of the rest representing products developed for small companies by private formula houses. In 1960 the seven classes of drugs most frequently prescribed in oral prolonged-acting form were anorectics, ataractics, antitussives, antihistamines, antispasmodics, cardiovascular agents, and hematinics. Of these, the anorectic preparations had a dollar volume more than twice that of any other type of oral prolonged-action drug and almost two-thirds of all antiobesity products were in this form. Forty per cent of the oral antihistamines were of the long-acting variety.

This review will primarily cover recent information about oral prolonged action drugs with reference to absorption from the gastrointestinal tract, methods of prolonging duration, procedures for *in vitro* and *in vivo* testing, and recent clinical evaluations. Several reviews (2–9) on oral prolonged-action drugs have already appeared and it is the authors' desire not to reiterate what has already been written, but to amplify on some of the gastrointestinal factors which influence absorption. Many approaches to the problem of prolonged action have not been explored in depth and these will be described. The

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long-acting dosage forms have been criticized, some justifiably, but many objections appear unwarranted. An attempt will be made to correct the misunderstandings.

GENERAL CONSIDERATIONS

The literature indicates that most of the fundamental studies on absorption are, as would be expected, conducted in hospitals and clinics associated with medical schools and academic institutions. Practically all of the participants in recent symposia (10, 11) on gastroenterology and the mechanism of absorption in the gastrointestinal tract were from academic institutions. Drugs are investigated pharmacologically by the manufacturing firms discovering them and only after thorough study in the animal are they investigated clinically. A few academic institutions investigate the pharmacological activities of new drugs in conjunction with industry but very few, if any, of the industrial research laboratories study the fundamental aspects of the mechanism of absorption. Brodie and colleagues of the National Institutes of Health Laboratories and several others are doing such studies on the mechanisms of drug absorption. The analytical problems involving quantitative measurements of the absorption of drugs and the physiological differences between man and laboratory animals have contributed to the difficulty of studying the absorption of drugs from the gastrointestinal tract. Many drugs are tried in animals and because of poor solubility or rapid degradation due to hydrolysis or oxidation may be rejected. A slight modification of structure or the formation of another salt or ester, based on an understanding of the physicochemical properties of the gastrointestinal tract, may render the drug more effective in the clinical situation. A better understanding of absorption can provide guide lines to the synthesis of new drugs with better therapeutic response.

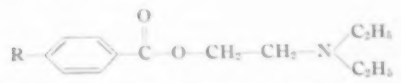
Drugs which are incorporated into long-acting dosage forms are generally those whose pharmacological properties, chemistry, therapuetic indications, and hazards have been well evaluated. The dosage form does not change the effect of the drug but is specially intended to alter the release of the drug to the fluids of the gastrointestinal tract.

The primary advantage of increasing the duration of action of a drug is to prolong its therapeutic effect, particularly where continuous action at an adequate level is essential for mitigation of the symptoms of the disease process itself. Infectious diseases, tropical and helminthic diseases require prolonged treatment, the length of treatment depending upon the elimination of the causative organism. The management of peptic ulceration demands continued drug action and in hypertension, longer acting ganglionic blocking agents, tranquilizing, and other hypotensive remedies are desirable. Antihistamines with long duration of action are required to afford persistent relief of allergic symptoms. The often repeated statement of convenience to the patient is important, particularly when the patient's sleep should not be disturbed. A constant and uniform release of drug to the body tissues in an amount sufficient to maintain a therapeutic level is possible with properly designed long-acting dosage forms. Of lesser importance and of minimum concern in administering long-acting drugs is the reduction in nursing time and the providing the consciously or otherwise forgetful with their medicament.

Rapidity of onset of action is important, and where the modification of a drug to prolong its action delays its onset and/or does not provide a satisfactory therapeutic level, the untreated drug is frequently included in the dosage form. Once the therapeutic level is obtained, the high and low concentration levels of the drug which occur with single dose administration are eliminated. When administered by mouth, prolongation of drug action may be obtained by reducing the rate of absorption, delaying its chemical degradation, inactivation, or excretion. The methods of delaying the chemical degradation, inactivation, and excretion will be discussed first.

Harper (12) has written an interesting review illustrating how some drugs have been chemically modified to alter their absorption, distribution, and enzymatic transformation. He termed this chemical approach to drug design as "drug latentiation" and defines it as "the chemical modification of a biologically active compound to form a new compound, which upon *in vivo* enzymatic attack will liberate the parent compound." "Structural formulation" also involves chemical modification of an active substance in order to alter its absorption, distribution, or metabolism but does not affect its biological response nor does it involve the liberation of the parent compound. Harper cites the work of Glazko, *et al.* (13), who prepared a number of chloramphenicol esters. These were poorly absorbed, but the rate of absorption appeared to depend upon the rate of enzymatic attack. The chemical nature of the fatty acid portion of the ester, the degree of physical subdivision of the substance, and the interfacial tension of the ester

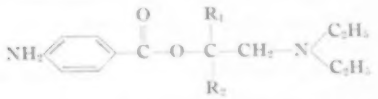
and water affected the rate of hydrolysis of the ester. Harper refers to the work of Levine and Clark (14) in discussing some basic concepts of drug latentiation involving the chemical transformation of an active compound into an inactive form for transport, after administration, to its site of action in the body where it is released by enzymatic activity. They have shown that a change in polarizability or ease in electron displacement of a functional group can alter the hydrolytic rate.



(A) R = F—rate of hydrolysis in human serum is 3,000 mcg. split/ml. of serum/hr.
(B) R = NH_2—rate of hydrolysis in human serum is 50 mcg. split/ml. of serum/hr.

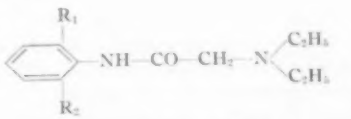
(A) was found to be hydrolyzed *in vitro* by human serum much more rapidly than (B) which has an amino group in place of the halogen. Enzymatic hydrolysis is thus influenced by the electronic character of the substituent group.

Steric hindrance and the configuration of the active drug moiety also play important roles in the rate of enzymatic hydrolysis. It has been demonstrated



	R_1	R_2	Rate
(C)	H	H	500
(D)	H	CH_3	15
(E)	CH_3	CH_3	0

in vivo that (C) was more rapidly hydrolyzed in human blood serum than (D) or (E), indicating the effect of steric hindrance due to the substitution of the methyl groups on the alpha-carbon of the alcohol. The prolonged duration of action of lidocaine (F) as compared to (G) and (H) has been attributed to the presence of two *ortho*-methyl groups.



(F) $R_1 = R_2 = CH_3$
(G) $R_1 = H, R_2 = CH_3$
(H) $R_1 = R_2 = H$

Increased stability of the amide linkage and resistance to acid hydrolysis by the substitution of the *ortho*-methyl groups accounts for the prolonged activity (15).

Attempts have been made to prolong the action of penicillin and *p*-aminosalicylate by controlling their excretion by the simultaneous administration of probenecid, but the inconvenience of maintaining a therapeutically effective level of the latter has limited its use in this manner. Drugs which are stored in certain tissues from which they are slowly released will have a longer duration of action. Chlorotrianisene is an oral long-acting estrogen by virtue of the fact that it accumulates in the body fat from which it is gradually liberated (16).

The rate at which a drug is inactivated may be modified by inhibiting the enzyme system responsible for its inactivation. The action of acetylcholine may be prolonged by the administration of neostigmine and other anticholinesterases which combine with the enzyme, cholinesterase, to delay the hydrolysis of acetylcholine. The effect of morphine on pain is diminished by atropine, indicating that atropine is antagonizing an acetylcholine-like action (17). The addition of neostigmine to morphine sulfate and methadone hydrochloride prolonged their duration of action and also produced greater sedation (18). A sustained-action effect can also be attained by the administration of substances which in themselves are almost inactive, but possess the ability to inactivate or inhibit enzyme systems in liver microsomes which act on other drugs (19). Iproniazid and other substances can interfere with the metabolism of barbiturates, amphetamines, and amidopyrine, thereby prolonging their effects.

A successful application of the principle of retarding absorption in the stomach has been applied to the development of the "cobalt bullet" (20). Sheep and cattle grazing on land lacking cobalt developed a vitamin B_{12} deficiency which often proved fatal. Prolonged action was obtained by feeding compressed cylindrical pellets containing cobaltic oxide in china clay to the animals. The specific gravity of the pellets was such that they remained in the reticulum and cobalt was released from them for several weeks. The idea of a drug repository in one of the compartments of the animal's stomach was based on the observation that stones and other small heavy masses tend to collect in the reticulum from which they are difficultly dislodged because of gravity.

PHYSIOLOGICAL FACTORS AFFECTING GASTROINTESTINAL ABSORPTION OF DRUGS

The absorption of drugs from the gastrointestinal tract has been the subject of many reviews. The physiological aspects of secretion

and absorption (21) were reviewed by Hogben as well as the mechanism of transport across the gastrointestinal epithelium (22). Brodie and coworkers (23–27) studied the absorption of drugs in the rat and in man and elucidated the concept that the barrier between the gastrointestinal lumen and the blood has the characteristics of a lipoidal membrane permeable to oil-soluble materials in an unionized form. In a review of their work, Schanker (28) stressed the classification of drugs as weak electrolytes and that absorption in the gastrointestinal tract of drugs is dependent upon two factors: the lipid-water partition coefficient and the dissociation constant of the substance. The oil-soluble, undissociated molecules of weak organic acids and bases are readily absorbed, while substances which are completely ionized, such as the quaternary compounds, are very poorly absorbed. A highly lipid-soluble substance like thiopental is absorbed readily and a poorly oil-soluble drug like sulfaguanidine is slowly absorbed. For moderately oil-soluble materials the picture is vague. The lipid-water partition coefficients were found to be high for drugs which are absorbed readily and low for the poorly absorbed drugs. Chloroform and heptane were the two solvents used to determine the coefficients. However, the relative order of solubility of the drugs in chloroform was not the same as the relative order of solubility in heptane. Wagner (29) suggests that the reason for the vague relationship between the partition coefficients and the rate of intestinal absorption for the moderately oil-soluble drugs may be that chloroform and heptane are not the proper solvents for the estimation of the partition coefficients. Hobgen, *et al.* (27), state that "until more is known of the chemistry of the intestinal blood boundary, there is no *a priori* basis for expecting that any one solvent would truly mirror the cell membrane."

In addition to lipid solubility, the ability of a drug to bind with protein may be an equally important factor affecting absorption. When a molecule contacts the cell membrane the molecule must adhere to the membrane before it can be absorbed into the cell. Schedl (30) studied the binding of a series of steroids to albumin and found a direct relation between absorption rates and protein binding, as determined by perfusing the small intestine of the intact rat. His studies also showed a direct correlation between the absorption of barbiturates and other drugs and protein binding. Absorption rates were found to vary inversely with hydroxylation, while acetylation increased the absorption rates. These data further support the concept of a lipoidal gut membrane.

The pH-partition coefficient hypothesis is apparently applicable to the passage of foreign substances through membranes of other organisms. It is known (31) that phenolic compounds are not bactericidal when dissociated, since the phenolate ions are poorly lipid-soluble as shown by partitioning between water and the lipid phase and are, therefore, not able to pass the lipid barrier of the bacterial cells (this barrier is probably located in the cytoplasmic membrane). It has been demonstrated with aqueous solutions of undissociated phenols and of aromatic alcohols (31) that the antibacterial activity is parallel to the molar concentration of antibacterial agents which is reached in a lipid phase after partitioning. With respect to these disinfectants, a suitable lipid phase may therefore serve as a model for the bacterial cell as well as for a crude comparison of their antibacterial activities. Similar observations have been made with other antibacterial compounds (32, 33) as well as with other biological systems, and all of these authors have stressed that the activities of the compounds examined are governed by the molar concentration reached in the lipid phase.

Based on the works of Teorell (34), Boxer (35), and Swintosky (36), Lazarus and Cooper (2) have attempted to explain the physiological factors which determine the attainment of a therapeutic drug level in the blood and tissues. The rate at which a substance enters the blood stream must be constant and continuous so that the amount absorbed per unit time would effectively control the concentration level. The pKa of the acid or base is a convenient value which can serve as an indicator of the extent of ionization or the amount of material which may be available for absorption. Albert (37) has discussed the calculation of the dissociation constants and their relationship to the pH and biological activity. In Wagner's excellent review, the various factors affecting absorption and the rate of absorption in the gastrointestinal tract have been covered (29). The kinetics of drug absorption, metabolism, inactivation, and elimination were thoroughly treated in Nelson's review (38). These aspects which are fundamental to a discussion on absorption will not be discussed here in detail in view of the adequate and appropriate treatment already accorded to them. However, some pertinent studies, which shed some light on this complex area, will be briefly mentioned.

Hogben (21) suggests that the diffusion of a

solute within the lumen may be the limiting step in absorption, since evidence has been accumulated to indicate that permeability of the mucosa can be excluded as a rate limiting step in absorption. He and co-workers (26) demonstrated that the rapid absorption from the small intestine, when normal motility is suppressed, is not limited by blood flow. Motility regulates the rate of transit of material in the gastrointestinal tract as well as the rate at which the material is exposed to a greater surface area. The finding that caffeine accelerates absorption from a Thiry Vella loop and that bromide depresses it may be an example of the effect of motility on absorption (39). The effect of emotions on motility in normal subjects, in patients with the irritable colon syndrome, and in patients with ulcerative colitis has been investigated (40). All three groups showed positive colonic response when subjected to an emotionally charged interview. In spite of many years of study, the effects of drugs on the activity of the small intestine are still poorly understood (41).

Gastric emptying is affected by the fat content of the meal. Turner (42) found that in rats the weight of fat in the intestinal lumen is controlled by the stomach which limits or releases the amount of lipid which can keep up with the absorption mechanism. There is no specific emptying of any one specific glyceride or fatty acid. A constant amount of fat is found in the proximal and distal portion of the mucosa. Although the amount of fat remaining in the stomach following a normal test meal steadily decreased over a four-hour period, the contents of the intestinal lumen remained the same. Turner, *et al.* (43), have postulated that the gastric regulatory mechanism may be related to the degree of saturation with absorbed fat of the intestinal mucosa and peripheral tissues. As the plasma becomes more turbid, the lymph becomes turbid, and the mucosal cells decrease absorption.

Drugs are presumed by Schanker (28) to cross the gastrointestinal epithelium by passive diffusion. Passive diffusion, passive transport, or passive absorption has been distinguished from active absorption or active transport by the ability of the latter process to effect absorption against a concentration difference or by requiring energy for the molecules to cross the membranous barrier. The energy may come from metabolic reactions or from the absorption of environmental heat (44). Since all molecules may be assumed to obtain equal energy from the environmental heat, in the absence of an additional supply of energy, the transport of the molecules occurs by diffusion in the direction of the lower concentration (45). The term active transport is used when metabolic energy is required for the transfer of molecules across a membrane and is restricted to their movement against a concentration difference. If a concentration difference exists between a nonionic substance in the intestinal lumen and the substance in the blood stream which has the higher concentration, absorption will be effected against this difference. In the case of ionic material, the driving force is usually the electrokinetic potential. Crane (44) suggests the possibility that active and passive absorption may not be two separate processes, but may actually share the same initial step. For example, the entrance of sugars into the cell may be energy-independent followed by an energy-dependent movement against a concentration difference (46). A suggestion has been made that a common pathway is involved in sugar transport, since in *in vitro* experiments the presence of one transported sugar inhibited the transport of the second (47).

Other transport mechanisms have been proposed for the absorption of water-soluble substances which pass the lipoidal membrane barrier in spite of their ionic or lipophobic properties. The existence of a special mechanism for the hexoses and another for amino acids has been recognized for some time (21).

Schanker and Tocco (48) have reported on another mechanism for active transport of pyrimidines and possibly for purines based on *in vitro* experiments with the rat's small intestine. A fourth and different mechanism is recognized for the absorption of vitamin B_{12} (49). However, these mechanisms do not account for the absorption of some of the water-soluble compounds such as the members of the vitamin B complex and ascorbic acid.

Proteins do not generally pass the lipoid barrier, but absorbed proteins have been detected in newborn mammals by immunological methods. Clark (50) has suggested that the absorption of proteins occurs by pinocytosis, a concept of a cell "drinking in" its surroundings. The rate of absorption occurring by this means is slow and may account for the limited absorption of intact proteins. The absorption of proteins, which is insignificant in the adult, must be an indirect process. If this were a process of simple diffusion, there would be an intolerable loss of plasma proteins (22).

The mechanisms of transport through biological membranes have been intensively studied not only from the viewpoint of gastrointestinal absorption, but also for biological systems in general. The theory of irreversible thermodynamics has been applied to them because it identifies the forces involved in a transport process (51). Miller (52) has applied this principle to show that the chemical potential gradients rather than the concentration gradients are the true driving forces for isothermal diffusion.

Ion exchange membranes have been used as inanimate models for the living membranes which have the characteristics of a well organized material about 50 to 150 Å. in thickness and are composed of proteins and lipid material having charged sites distributed in aqueous and nonaqueous channels. "The active transport mechanism derives its energy from metabolic processes and presumptively achieves its specificity from the binding constants of the interaction between ion and charged site, the dielectric constant of the medium in the channel, the hydrated and unhydrated radii of the passenger ion, and the dimensions of the channels for passive and active transit" (51).

Equipment has been designed to permit the release of drugs at various sites in the gastrointestinal tract so that absorption may be related to the site of release (53). The available techniques for studying absorption such as the Miller-Abbott tube, the gastrointestinal fistula, and variations of these procedures create abnormal conditions in the test subject which may influence the results. In the nonsurgical procedure a radio frequency generator is used to open a specially designed capsule at various sites in the gastrointestinal tract without direct connection of the capsule to equipment outside the body. The technique is of importance in the evaluation of drug absorption, since it may be used to determine the relative areas in the tract where absorption of a particular drug occurs.

A study was made, using dogs, on the rate of absorption and the efficiency of absorption of salicylate ion following its release at several sites in the gastrointestinal tract. Since the drug continues on its way through the tract after its release from the capsule, the absorption data do not represent the absorption from a particular site, but rather from an area extending from the site of release to the point where absorption ceases. The amount of drug absorbed was measured by its concentration in the blood. The study showed similar absorption of salicylate ion over the first half of the small intestine. Poor absorption was noted in the cecum. The frequent shocks the dog received during the test did not appear to affect absorption propensity (54).

The development of electronic instrumentation for transmitting information about the gastrointestinal tract has increased our potential for studying its physiology. A radio frequency heating device has been reported for recovering samples from the gastrointestinal tract (55). The motor action of the stomach, small intestine, and colon can now be studied by means of an ingestible, pressure-sensitive radiotelemetering capsule (56). The pressure and motility in the hitherto most inaccessible areas of the gastrointestinal tract, such as the ileum and the ascending colon, can now be recorded. The capsule measures the contraction of the gut, but since it measures only one parameter of motility it cannot provide a complete picture of intralumenal pressure. One of the disadvantages of the capsule was the limited life of the tiny, replaceable battery. A similar capsule, termed an "endoradiosande," was developed independently in Sweden. The battery is said to have a "life" of seven days (57). The various pressure patterns in the lower esophagus have been recorded by combining cinefluorography with pressure detection (58).

Just as the mechanisms of gastrointestinal absorption are vaguely understood, the mechanism of drug action is frequently not known. Paton (59) states that the principles of drug action can be divided into two large areas. The first would concern itself with the fate of the drug such as its absorption from the site of administration, distribution, metabolism, its exclusion from or concentration in certain body tissues, and its elimination. The other part would concern itself with the kinetics of these variables. In the absence of special transport mediums, such as may be required for the absorption of sodium, potassium, calcium, glucose, and iron, fat solubility is important. Fat solubility decreases with ionization. When ionized material is absorbed it remains in the extracellular fluid because it cannot penetrate the cells which, in turn, prevents its metabolism. In the kidneys, it is filtered but not reabsorbed or secreted since it does not enter the cells. This is the distribution pattern of hexamethonium and streptomycin (both bases) and heparin (which can be considered an acid).

Paton has added a third component to the understanding of drug activity in the body. In addition to absorption and the kinetics of drug distribution, the mechanism for the mediation of

the response is not known. An accepted explanation of drug action has been the postulation of the existence of specific receptor sites which a drug occupies with the response to a drug dependent on the proportion of receptor sites occupied. With this hypothesis, it is possible to apply Langmuir's isotherm, a mathematical expression for the adsorption of gases on metals.

Paton has suggested that it is not the occupation of a receptor site which excites, but that the reaction with the receptor itself is responsible for the excitation. Once the union has been established, no further excitation takes place and no activation occurs until the receptor is freed by dissociation of the drug. If the dissociation is hindered then antagonism exists. This theory involves the kinetics of the reaction which is dependent upon the dissociation constant of the substance from the receptor site.

Although the theory of the mode of action of drugs is not directly related to their duration of action, a comprehension of a drug's action will permit the development of suitable mathematical models with fewer assumptions for use in designing a dosage form. The biggest problem at present appears to be an analytical one rather than the lack of mathematical models. While the equations are available, little information is in the literature relative to data required for use in the equations. For example, much has been written on sulfa concentration in body fluids. This is because the sulfa drugs are administered in large quantity and their method of detection is simple. Satisfactory bioanalytical methods are not yet available for many other drugs, including the antihistamines, as evidenced by their absence in the appropriate literature. Without the necessary data the mathematical models may be compared to a grounded deflated weather balloon which has the enormous potential to relate information but lacks the means of ascent. New and improved analytical techniques for evaluating drugs in body fluids will enable the models to ascend to the point of utility by the industrial and academic researchers.

The brief discussion of some of the aspects of gastrointestinal absorption was intended to highlight the complexity of the problems involved in designing long-acting dosage forms. To understand the nature of the variations in absorption observed in individuals and within the same individual, it is necessary to gain more knowledge about gastrointestinal physiology and gut mucosal biology. The protective or "barrier" function of the mucus of the digestive tract is not too well known. The protective effects are probably due to the chemical antibacterial and antiviral actions of mucus and partly to its mechanical properties (60). The role of intestinal bacteria, hormones, including histamine and serotonin, the constituents of the diet, the constituents and physicochemical properties of the gastrointestinal secretions, and the effect of the autonomic nervous system in adjusting cellular metabolism and motility are little understood. The concerted and integrated effort of individuals trained in different disciplines is required for the solution of the problems in many of these areas (61).

PRINCIPLES OF DRUG RELEASE

In spite of the limited available knowledge concerning the physiology of drug absorption, effective oral prolonged-action drugs with a duration of activity of eight to twelve hours have been developed. Drugs which are inherently long-acting when taken orally are usually effective independent of dosage form. Chemical modifications of a drug to prolong its duration of action in man is not always feasible or practical. Continuous or slow release of the active substance is accomplished by manipulations of the dosage form or by complexing. A physical barrier is used in the majority of the long-acting preparations to decrease the rate of release of the drug to the gastrointestinal tract. Many drugs have been absorbed onto ion exchange resins or complexed with high molecular weight colloids.

The long-acting preparations have been classified according to the method of manufacture: coating of the drug, embedding the drug in a wax-fat vehicle or porous plastic base, binding the active substance to an ion exchange resin, or complexing with colloidal material. The general methods of manufacture of the prolonged-action preparations have been described in reviews and in the patent literature (62). A new type of oral sustained action preparation, the Duretter[1] tablet, was recently described (63).

The Duretter tablets are produced by compressing a granulate of the active substance with an insoluble, inert plastic material. Release is achieved by dissolution of the drug particles and is dependent upon the area of the interface between the plastic and the dissolving medium, the thickness of the diffusion layer which is the saturated solution formed at the interface between the solid and the liquid, and the difference in concentration between the diffusion layer and the solution in general. The release of a sparingly soluble drug

[1] Fryklöf, Sandell, and Östholm, British pat. 808,014.

can be increased by the addition of a soluble substance. When all of the drug has dissolved or leached out, the insoluble plastic skeleton still retains the original shape of the tablet. The release pattern may be controlled by varying the proportions of soluble and insoluble substances, forming multilayer tablets, or compress coating the plastic material around a core of the drug granulate. The advantages claimed for nonpan coated prolonged-action preparations exclusive of the ion exchange resins and chemically complexed substances are homogeneity of dispersion and sharp control of the weight of the active substance as achieved by mechanical means. Although the precise means by which drugs are released from long-acting preparations have not been clearly demonstrated, the claimed principles upon which the release is based were recently reported by Cooper (64) and will be discussed for a representative number of tablets. This information was obtained by personal communication with manufacturers of prolonged-action products.

In the Timespan[2] tablets release of the active drug is based upon delayed diffusion from a physiologically inert matrix. The amount and composition of the inert matrix controls the rate of release. The equivalent of three normal doses are embedded in the base. One dose is released within an hour after swallowing while the other two are released over a period of six to twelve hours. The Novahistine[3] L.P. tablet utilizes the principle of slow penetration of moisture into the tablet with gradual flaking off of the surface to obtain prolonged activity. Since it is based upon wetting action, pH is apparently not a variable. A two-layer tablet is used to obtain prolongation of activity of Peritrate.[4] One layer slowly disintegrates releasing 60 mg. of drug over an eight-hour period. A twelve-hour therapeutic effect is claimed, since activity is said to persist for four hours after the drug has been released.

Pan coating is used in Extentabs[5] to apply one-third of the drug onto the core containing the other two-thirds of the total drug content. The coated material is released immediately while the rest of the drug is gradually released to the intestinal fluids. Spacetabs[6] are compressed from a mixture of specially prepared release-retarding granules and granules made in the conventional manner. The untreated granules release the drug initially followed by slow release from the release-retarding granules. The Gradumet[7] tablets are somewhat similar in principle to the Duretter tablet and are prepared by mixing the active drug with inert plastic material and compressing. To facilitate the release of the drug, an excipient is added to the drug to draw fluids from the gastrointestinal tract into the tablet. The plastic matrix is insoluble and retains its shape throughout its passage in the alimentary canal. The release of the drug is claimed to be independent of the pH, enzyme concentration, and individual variations in gastrointestinal motility.

The Lontabs[8] are compressed coated tablets containing some of the drug in the outer coat from which it is released immediately after swallowing. More of the active substance is uniformly dispersed in a base containing high molecular weight fats and waxes which release the drug by a process involving erosion and leaching. According to X-ray studies the core does not disintegrate, but gradually decreases in size.

The coatings used for preparing coated pellets are of two types (65). The pellets in the Spansule[9] type capsule are coated with lipids while the Medules[10] are coated with a polymeric film. Sustained action in the latter is dependent upon the pH of the gastrointestinal contents and stomach emptying time. The release of the active substance from a Spansule is primarily controlled by moisture vapor permeability of the lipid film. The drug, the composition of the coating material, and the thickness of the coating will determine the rate of moisture permeability. It is essential to have a uniform coating free of pitting, imperfections or flaws, otherwise the release rate may be altered by premature rupture of the coating. The pellets should also be of uniform size to avoid stratification of different particle sizes in the capsule-filling operation, and to make certain that the drug, per gram of pellets, is uniformly distributed.

The ion exchange resin drug complexes are usually available as capsules or liquid suspensions. "The dissociation of reversible complexes formed by certain ion exchange resins with organic acids and bases proceeds at a finite rate which can be controlled to produce a uniform release of the drug." The amphetamine resinate in capsule form[11] is claimed to exhibit these characteristics (66).

Drugs with an amine group in the molecule have

[2] Trademarked name of Roche Laboratories.
[3] Trademarked name of Pitman-Moore.
[4] Trademarked name of Warner-Chilcott Laboratories.
[5] Trademarked name of A. H. Robins & Co.
[6] Trademarked name of Sandoz Pharmaceuticals.
[7] Trademarked name of Abbott Laboratories.
[8] Trademarked name of Ciba Pharmaceutical Products, Inc.
[9] Trademarked name of Smith, Kline and French Laboratories.
[10] Trademarked name of Upjohn Co.
[11] Marketed as Biphetamine resin by Strasenburgh.

been complexed with tannic acid. This large molecular polyionic complex is the basis of the Durabond[12] principle which is claimed to release the drug gradually and uniformly, independent of intestinal motility or pH, and without the use of waxes, resins, or enteric coatings (67).

The preparation of ion exchange resin complexes of riboflavin 5-phosphate and penicillin V and their *in vitro* and *in vivo* release rates have been described by Brudney (68). He stated that several factors, which may be readily controlled, affect the release of the drug from the resin. These include the particle size of the resin, the degree of cross-linking of the resin, the temperature during elution, and the quantity of the drug removed from the particles during elution.

An aqueous suspension of methapyriline resin complex was formulated and its pharmacological activity was compared with the untreated drug. The activity of the drugs was measured as a function of their ability to protect the intact guinea pig against histamine-induced asthma. The experiments demonstrated that the resinate was at least equal to if not better than a solution of the pure drug. Statistically, the difference was not significant (69).

The development of a long-acting dosage form must be preceded by a thorough knowledge of the drugs' action both in laboratory animals and in man. Drug activity and its duration are usually measureable by physiological response and/or blood or tissue levels. The interest in prolonging duration of activity has introduced the concept of the "biological half-life" of the drug as a factor in estimating the amount of drug to be included in the finished long-acting form (70). The half-life factor is suitable wherever quantitative measurements can be made of blood level concentrations or of physiological response. However, no half-life data has been published on antihistaminics and other drugs where such measurements are impractical because of bioanalytical problems. Equations have been developed which permit the calculation of the amount of drug needed to provide the desired duration of action (36, 71–75).

IN VITRO TEST METHODS

During the development of the dosage form, the *in vitro* test serves as a guide in estimating the amount released per unit time. Once correlations have been made by animal and clinical studies in man, the *in vitro* test serves as a quality control procedure. Reproducibility of the release data in the *in vitro* test is an indication that the manufactured batches meet the specifications set for the dosage form and that the therapeutic effects will be similar regardless of batch.

Recognition must be given to the fact that prolonged-action dosage forms are not only drugs but also devices, and therefore "no single *in vitro* test will completely reflect the availability of the drug" (1). The usual tests of disintegration time found in the pharmacopeias do not apply to oral prolonged-action dosage forms, since the release rate per unit time is the critical factor. The equipment and procedure for measuring release rate varies with the manufacturer, and some methods employed have been reviewed by Lazarus and Cooper (2).

The mechanism of release of the drugs from various dosage forms is different for each form. The ion exchange adsorbates are said to be independent of pH; some of the encapsulated pellets have coatings which are pH-dependent, while others are said to be dependent upon moisture vapor permeability. In view of the wide divergence of mechanisms, one standardized mechanical unit and eluting or immersion fluid has not yet been developed and may prove to be an impossible task.

Seven *in vitro* methods were reported between 1956 and 1958 and were reviewed (2). Three more were published in 1959 and these will be briefly described. Shenoy, *et al.* (76), used a modification of the "replacement closed tube" method for determining the release of eight encapsulated pelleted amphetamine preparations of the sustained-release type. The contents of two capsules were placed into a 4-ounce bottle containing 100 ml. of immersion fluid and rotated end over end at 12 r.p.m. U.S.P. XV simulated gastric fluid was used for the first thirty minutes followed by U.S.P. XV simulated intestinal fluid for the rest of the test interval. Filtered aliquots of 20 ml. each were withdrawn every fifteen minutes for the first hour, and then at two, three, four, six, and seven hours after the start of the test and replaced with 20 ml. of fresh fluid. The extracts and not the residues were assayed.

An apparatus has been designed for *in vitro* screening of experimental formulas of long-acting preparations and for control procedures (77). The test sample is placed into 400 ml. of fluid contained in a 600-ml. Buchner-type funnel with a medium porosity fritted disk fitted to a suction flask having a two-way stopcock fused to its bottom. All operations are run at room temperature. The fluid and sample are mildly agitated

[12] Trademarked name of Irwin-Neisler.

with a variable speed stirrer. A 200-ml. quantity of fluid is drawn off at intervals by vacuum through the filter into the suction flask and collected in a graduated container with 200 ml. of fresh fluid, replacing the withdrawn quantity. Samples are taken one-half, one and one-half, two, three, five, seven, and twenty-four hours after the start of the test. Simulated gastric fluid is used for the initial one and one-half hours following which simulated intestinal fluid is added. Drugs with limited solubility may require more frequent changes of the immersion fluid, and with some sustained-action products it may be preferable to change the pH gradually. Screen sizes may be varied if the preparation disintegrates into fine particles.

In a collaborative study, several laboratories studied three different long-acting products using a modified U. S. P. XV disintegration apparatus. The basket-rack assembly was replaced with a holder for six stainless steel cartridges closed at both ends with a 40-mesh screen. U. S. P. XV simulated grastric fluid, maintained at a temperature of 37 ±2°, was used as the immersion fluid for the first hour, followed by U.S.P. XV simulated intestinal fluid for the rest of the test period. Assays were run for the drug in the extracting fluid as well as the residue and no significant difference in the results was noted (78).

Another collaborative study was undertaken and a progress report has been published (79). Four samples were tested by each of the laboratories participating in the collaborative study. The assays were conducted on the residues in order to minimize interference from other substances present in the test fluids. Each sample was assayed by three different *in vitro* techniques: the cartridge and basket method (78), rotating bottle method (80), and the FDA method (2). Two procedures were followed with the first two methods: (*a*) an abrupt change in pH after one hour followed by a complete change in bath fluid from simulated gastric fluid to simulated intestinal fluid, and (*b*) a gradual change in pH by withdrawing at hourly intervals one-half of the test fluid.

All laboratories reported similar results on the four samples. One sample contained a freely soluble drug and the other three had sparingly soluble drugs. The freely soluble drug gave good results with the rotating bottle, cartridge and basket, and the FDA methods. The sparingly soluble drugs gave good results, in all laboratories, with the rotating bottle method and poor results with the other three procedures. The difference in results appeared to be due to the greater flow of fluid around the tablets and capsules in the rotating bottle method.

The *in vitro* test serves to reduce the number of samples considered suitable for *in vivo* testing during the developmental stage. For these preliminary studies or screening tests simulated pH changes and a temperature of 37° are not considered mandatory, since only very special information is sought at this stage (78). *In vitro* data have significance only when they can be quantitatively correlated with *in vivo* data (81, 82).

Wagner subjected the published *in vitro* data on the release of drugs from prolonged-acting products to mathematical treatment and found that most drugs are released at pseudo first-order rates. The rate of release in simulated gastric fluid differed from that in simulated intestinal fluid. The advantages of such kinetic treatment of the data are many. The drug release results obtained with different *in vitro* test methods on the same products can be compared as well as the results with different batches of the same material. The linear equation which he derived allows the calculation of the instantaneous rate of drug release from the dosage form at any time during the test. Another important advantage for the kinetic treatment of release data is that the calculation of statistical confidence intervals for the rate constants permits a reduction of the number of assays required for production control provided, of course, that the validity of the *in vitro* test procedure has already been established by comparison with a reliable *in vivo* method. When the confidence intervals are established the normal variations to which every product is subject in the development stage, production, and analytical areas must be considered (83).

Wiegand and Taylor (84) have also found that the rate of release of different drugs contained in a variety of long-acting dosage forms may be estimated from the same first-order equation, which they derived, even though the rates of release and the fractions of the dose finally released are not the same. Their equation may also be used to predict *in vivo* response in terms of drug concentration in the blood and tissues, if the *in vitro* and *in vivo* release patterns are the same. They also found a strong correlation between the apparent first-order constants for absorption and disappearance of the drug from the plasma of dogs and the *in vitro* release constants for a sustained-release tablet obtained by *in vitro* testing (85).

Münzel (86) reported on similar equations for the calculation of the release rate and other constants from the analytical data for drug release following a zero order and first-order pattern. These constants will vary slightly for each batch, but after several batches have been run and these constants determined, the statistical 95% confidence limits can be calculated. It must be remembered that the values used for establishing the confidence limits are obtained only from batches of the prolonged-acting dosage form which have the desired clinical effect. According to Münzel, only two determinations are necessary once the rate constants and their 95% confidence limits are established, thereby reducing eight hours of testing to two hours.

The release rate pattern should be checked frequently since very slight manufacturing changes may alter the release of a drug from an apparent zero-order rate to an apparent first-order rate, changing the significance of the constants. The recommendations are similar to those of Wagner (83).

IN VIVO PROCEDURES

The direct measurement of the duration of the desired therapeutic response of a drug is unquestionably the best method of evaluating the *in vivo* release properties of a long-acting dosage form. Unfortunately, very few drugs can be directly evaluated in man since the response cannot always be quantitatively measured or subjected to statistical treatment.

Several *in vivo* techniques have been used for estimating duration of drug activity. The measurement of drug concentration in the blood affords a means of estimating the time of onset and estimation of the duration of effect of a drug provided the therapeutically effective minimum and maximum blood level concentrations have been determined. Availability of the drug cannot be quantitatively related to blood levels at various times unless the area under the blood level curves or some other criteria is used to relate response to dose (87). Using prednisolone as a tracer, Wagner, *et al.*(88), measured the plasma 17-OHCS levels in man and dog of two slowly releasing dosage forms and for fairly rapidly releasing forms. A study was also made of their *in vitro* dissolution rates. The long-acting dosage forms differed from one another in that the granules of one were coated with a pH-dependent material while the coating on the other was pH-independent. The blood level curves for the slowly releasing forms showed no significant difference, but was different for the rapidly releasing forms. The plasma levels for the long-acting forms were sustained for twelve hours in man (based on averaging the values obtained on 10 subjects) and only seven hours for the conventional form. Since the areas under the curves were approximately the same for the coated granules, full availability was indicated. It would have been of interest to note the shape of the plotted curve following the administration of the conventional form in divided doses instead of all at once (89).

The serum levels following administration of penicillin V complexed with an ion exchange resin were compared with published results for uncomplexed penicillin V and penicillin G. The results indicated that the complexed penicillin gave a sustained level of penicillin for three hours and then decreased. The uncombined penicillin compounds gave high initial blood levels and then decreased rapidly (68).

The disadvantage of blood level determinations as an *in vivo* measure of the duration of activity is that a dose larger than the therapeutic dose must frequently be given to facilitate the chemical analysis. Moreover, many drugs cannot be detected in the circulating blood stream because of the bioanalytical problems which are involved.

Urinary excretion studies have been used as an *in vivo* technique to evaluate long-acting dosage forms. A direct relationship for certain drugs has been shown between excretion rate and the amount of the drug in the blood (90). Since the clinical response of many drugs is based on their concentration in the blood stream, it would appear that a valid relation exists between clinical effect and the amount excreted in the urine. To complete this syllogism, if a dosage form is found to have sustained release as evidenced by prolonged urinary levels, it is logical to conclude that it would have a prolonged clinical effect. Campbell, *et al.* (90), proposed criteria for the evaluation of sustained-release preparations of drugs which are detectable in the urine. The drug in the sustained-release form should exhibit a relatively constant urinary excretion rate at about the same level which is reached by a single dose of the drug. The rate should be maintained for the period for which sustained release is claimed. The validity of these criteria was established for amphetamine. Marked differences in excretion rate of creatinine, acetylsalicylic acid, and amphetamine sulfate complexed with ion exchange resins to provide prolonged duration were found (91). The rate of urinary excretion of the drugs after single doses of the resinates was compared with the rate

of excretion following doses of the pure drugs. One amphetamine resinate exhibited sustained action while the others did not. An interesting point was raised by these workers. They noted that a sustained-release preparation of the resinate type which they judged ineffective on the basis of urinary excretion tests had been claimed to be clinically effective and had demonstrated sustained release *in vitro* (92). It appeared that the criterion used to evaluate the clinical effects was not critical enough. However, when a quantitative measurement of the clinical response was employed (93) the results correlated with the urinary excretion data.

Also, on the basis of urinary excretion studies, Shenoy, *et al.* (76), found that claims of sustained action could not be justified for about eight different amphetamine preparations in pelleted form. Only two showed constant urinary excretion at an adequate level and were considered to be quantitatively available. According to their findings, it may be possible to calibrate existing *in vitro* test methods to correlate with *in vivo* results.

Urinary excretion studies have been used to evaluate drug absorption of aspirin, benzyl penicillin, sulfaethylthiadiazole (94), and tetracycline with mucic acid (95). It is pointed out that an understanding of the rate-limiting nature of the *in vivo* solution step is necessary when a change in the dosage form is considered. The data obtained from excretion studies on sulfaethylthiadiazole enabled the calculation of its absorption rate which was found to be in good agreement with published blood level data.

Good correlation between the *in vitro* release and urinary excretion rate was reported for a prolonged action nicotinic alcohol tablet (96). Physiological availability of nicotinic alcohol was determined by measuring the total excretion of all three nicotinic alcohol metabolites after ingestion of a conventional tablet and a prolonged-action tablet.

A comparison was made between the urinary excretion of trimeprazine and its metabolites administered in the form of six 5-mg. sustained-release capsules and an equal amount of unprocessed drug given in three divided doses every four hours. The total amount excreted was comparable, indicating physiological availability (97). Another comparison was made on the releasing properties of phenylpropanolamine hydrochloride contained in a sustained-release capsule and the drug in the untreated form by means of urinary excretion studies. The results indicated that the treated drug was excreted in a manner similar to the unprocessed drug when an equal dose of the latter was given every four hours for three doses (98). Urinary excretion studies and the measurement of drug disappearance from blood can facilitate the design of sustained-release dosage forms when blood level and excretion data can be related to therapeutic response (73, 74).

Toxicity studies have been used as an *in vivo* method of demonstrating duration of effect. Becker and Swift (99) determined the LD_{50} of several drugs in the form of the pure chemical and as ion exchange resinates. They noted that the lethal dose was significantly raised and the time of death (LT_{50}) was longer with the resinate forms. Their tests also showed that the larger particle resinates (beads) were superior to the finer particle resinates with the same drug strength in respect to LD_{50} and LT_{50}. The difference in lethal time and lethal dose were attributed to slower absorption from the resinate forms. No indication was given as to the physiologic availability of the drug.

Utilizing this technique, a comparison was made between the LT_{50} and the urinary excretion studies on two drug resinates (100). The data indicated a close correlation between the delay in the time of death and urinary excretion rate. The method appears to be a useful technique for the study of prolonged-action preparations containing relatively toxic drugs which cannot be evaluated by blood levels or urine studies in man. Toxicity assessment can distinguish between the pure drug given in solution or suspension and the long-acting product. However, when the response differs from the standard, one cannot tell readily whether or not the difference is due to the prolonged effect or to lack of physiological availability.

The physiological availability of riboflavin from five commercially available sustained-release vitamin preparations was determined on the basis of urinary excretion tests (101). No sustained urinary excretion of riboflavin was obtained nor could full availability of the vitamin be demonstrated in the products. The peak excretion occurred two to four hours later than the peak obtained when conventional rapidly releasing tablets containing 5 or 10 mg. of riboflavin were given. The excretion curves dropped at about the same time as the standard, and no sustained effect could be demonstrated. However, a sustained effect was obtained when riboflavin was given at two-hour intervals. The per cent excreted with this dosage was the

same as that when the same amount of drug was given as a single dose. Riboflavin is apparently absorbed in the upper part of the intestine or is partially destroyed in the lower bowel, since it was shown that it was possible to maintain sustained urinary excretion for only approximately two hours after the last dose of riboflavin in solution. It has also been shown that in growing rats the administration of riboflavin once daily by stomach tube resulted in similar weight gains as when the vitamin was given in the diet (102). These results indicate that a sustained-release preparation of riboflavin is unecessary and unwarranted.

An *in vivo* control procedure has been reported for use with prolonged-action products of the coated pellet type (103). The dye, fluorescein, is incorporated into the protective coating and when the coating is permeated or ruptured, the dye is released. Fluorescein was selected because it is pharmacologically inert, absorbed rapidly enough to appear in the urine two to ten minutes after ingestion, and easily applied in the coating procedure. *In vitro*, it was found that the quantity of fluorescein which was liberated corresponded to the quantity of drug liberated. The test drug was salicylic acid. Similarly, the fluorescein blood levels corresponded to those of salicylic acid in the *in vivo* tests. This test may be applicable to coatings that are free from imperfections for drugs that are absorbed readily. It would be of interest to know whether or not a correlation would exist between the dye and a slowly absorbed drug.

A unique *in vivo* technique involving an instrument, the Performance Indicator (104), has been developed which measures reaction time quantitatively in humans. It has been used to study the effect of alcohol (bourbon), amphetamine, and a nonamphetamine appetite depressant. The instrument measures precisely the amount of time required by the subject to respond to a light source stimulus applied at varying intervals. Since perception of the stimulus and the coordination of the muscles for response are essentially those of the central nervous system, the variation of the test individual's response can be used as a measure of the effect of a drug upon these reactions. A sustained clinical effect for an amphetamine ion exchange resin complex has been demonstrated with the aid of the instrument. A comparison was also made with uncombined amphetamine phosphate and a placebo (105).

Absorption of sustained-release oral amphetamine products in the rat was evaluated by recovery of the nonabsorbed material from the gastrointestinal tract. The rate of disappearance from the gastrointestinal tract was considered to be directly related to the absorption rate as determined on the soluble salt, coated granules of the salt, and ion exchange resin complexes. Amphetamine sulfate may affect stomach emptying time since the pure drug, the amphetamine resin complex, and the coated granules of amphetamine sulfate were found in the stomach eight hours after oral administration. A smaller percentage of drug was found in the intestine indicating more rapid absorption. In both the dog and the rat, the ion exchange resinate gave more prolonged blood levels (106).

An *in vivo* method using tritium-labeled *d*-desoxyephedrine combined with two types of cation exchange resins has been described (107). The resins were administered in capsules to dogs. The carboxylic resin released the drug rapidly in the stomach while the sulfonic acid resin released it slowly. In the intestine the rate of removal was about the same for both. The amount absorbed was determined by measuring the tritium levels in blood. The experiment demonstrated the suitability of the radioactive isotope as a tracer in animal studies.

Swift (108) studied the duration of effects of resin-loaded antihistamines in guinea pigs using a histamine aerosol technique for the production of experimental asthma in the animals. Four antihistamines were evaluated: pyrilamine, phenyltoloxamine, tripelennamine, and chlorpheniramine. Particle sizes of the resinate appeared to be very important in the duration of effect of the drug. Satisfactory protection could not be obtained with a granular resin complex of phenyltoloxamine or pyrilamine. However, both powdered forms of the resinate gave good protection and a prolonged effect. A satisfactory prolonged effect suitable for guinea pigs could be obtained by combining two resinates, but the administration of one of the above mentioned resinates did not provide adequate protection for a long enough period at a suitable dosage level.

CLINICAL STUDIES

In the past two years, there has been an increase in the use of objective criteria in the clinical evaluation of prolonged-acting preparations. The galvanic skin response test was used to follow the duration of action of the prolonged form of propantheline bromide. The conventional tablet and the long-acting tablet showed a significant difference from the placebo. The

conventional tablet appeared to lose its effect before the prolonged drug, but the difference was not statistically significant. A much larger dose than normal had to be used to elicit a suitable response (109).

The decrease in vasodilation and cholesterol levels were criteria in a study on sustained-release nicotinic acid tablets. Three out of ten patients had to discontinue use of the drug because of side reactions (110). Serum quinidine concentrations were measured on hospitalized patients with various cardiac arrhythmias after administration of quinidine sulfate U. S. P. and a long-acting quinidine preparation. Adequate serum levels were produced with the long-acting product. The dosage schedule for the long-acting form was considered more convenient than the schedules required with the conventional tablet (111).

The constant suppression of the size of a histamine-induced wheal in ten subjects served as an indication that the *in vivo* release from a triple antihistaminic in Dospan[13] tablets was gradual, uniform, and sustained. A comparison was made with an unidentified antihistamine in a repeat-action tablet and another unidentified antihistamine in the form of encapsulated coated granules. The release rates of the three forms were evaluated by an *in vitro* test (112).

The degree of nasal obstruction due to nasal polyposis was a parameter used to evaluate a mixture of prophenpyridamine maleate, pyrilamine maleate, and phenylpropanolamine,[14] in combination with 10 mg. of hydrocortisone. A reduction in the degree of obstruction was noted, but upon discontinuation of the drug, the polyposis returned. The tablets were administered three times a day, since half of the active substances are contained in the core which disintegrates (assumedly determined by an *in vitro* test) in three to four hours. The hydrocortisone was not in the prolonged-action core. In the same study, symptomatic relief of chronic allergic rhinitis was observed, but the combination of the antihistaminics, vasoconstrictor, and hydrocortisone was not effective in chronic asthmatic patients with moderate to severe disease (113).

A quantitative method was used for assessing antitussive activity of several drugs. Pneumotachographic tracings recorded the differences in both degree and duration of effect on the cough response induced by citric acid aerosols. The method enabled the investigators to classify ten drugs into three categories: those which had a sustained antitussive action for four hours or more, those with an effective action up to two hours, and those which were ineffective. Sustained cough suppression over the four-hour period was obtained with morphinolinylethylmorphine and dihydrocodeinone resin. The study was conducted on healthy subjects. Incidence of adverse side effects could not be evaluated by this method nor could the clinical effectiveness in chronic lung disease be determined (114).

The numerical rating by patients of the degree of pain relief afforded by several analgesics enabled investigators to rank the drugs statistically in their order of clinical effectiveness in suppressing pain (115). Analysis of the gastric contents in patients suspected of hypersecretion served as a quantitative measure of the effectiveness of an inherently long-acting anticholinergic, isopropamide, combined with sustained-release pellets of prochlorperazine. In normal secretors, no decrease in secretion was observed, but in hypersecretors the anticholinergic and the anticholinergic ataractic combination decreased the hydrochloric acid secretion (116).

A prolonged-acting tablet of belladonna alkaloid combined with phenobarbital was administered to twenty-three patients who had their gastric secretions aspirated before and after medication. Before medication, aspirations were carried out following an eight-hour fast period. The free and total acidity was measured. The authors reported that the free acidity, based on averaged values, was significantly decreased after swallowing the tablet when compared to the control period. However, three subjects showed no change, ten showed an increase, and ten showed a decrease in average acid content. Clinical evaluations were based on the subjective responses of the patients. The poor results, measured in terms of alleviation of symptoms, were confined to cases having a "functional overtone" such as irritable bowel, gastritis, and diverticulitis (117).

No comparison was made with a conventional dosage form given three times a day. The control of complaints did not coincide with the gastric secretion and the motility studies which were made. It would have been of interest to know whether the symptoms in the unsatisfactory cases could be controlled by adjusting dose to individual requirements.

Relief from pain as well as the reduction in the amount and frequency of narcotics needed to

[13] Doxylamine succinate with pyrilamine and pheniramine maleates as Tridecamine Dospan tablets by The Wm. S. Merrell Co.

[14] Marketed as Triaminic by Smith-Dorsey.

quell pain in ureteral colic were the criteria used by clinical investigators (118) to measure the effectiveness of atropine tannate. Side effects of atropine administration were present, but were not considered as objectionable as those resulting from a full dose of a narcotic. Atropine sulfate is short-lived when ingested orally and because of its short activity its beneficial effects are considerably reduced in patients with renal and biliary colic. Many undesirable side effects result from the oral administration of a dose sufficient to attain satisfactory therapeutic activity. The terms prolonged action or sustained release have been associated in the literature with a therapeutic effect obtained over a period of eight to twelve hours. Though prolongation of the action of atropine up to four hours appears to have been achieved, the term long-acting used in this connection is a relative one.

Many clinical studies lack objectivity primarily because the nature of the disease is such that it is difficult to make quantitative measurements. Subjective clinical studies have been reported recently on many oral long-acting preparations: a triple antihistamine (119), an analeptic combination of pentylenetetrazole and nicotinic acid (120), a long-continued-action analgesic (121, 122), a long-acting antacid-anticholinergic preparation (123), timed-disintegration capsules of pentaerythritol tetranitrate (124), a nonamphetamine anorectic agent in the form of an ion exchange resin complex (125), a long-acting antihistamine-decongestant combination tablet (126, 127), and a prolonged-acting belladonna alkaloid with phenobarbital (128).

In another study on a long-acting quinidine preparation for the reversion of atrial fibrillation, good results were reported but no comparison was made with a conventional tablet or placebo (129). Relief of morning stiffness in rheumatoid arthritic patients was considered an effect of a long-acting form of methylprednisolone (130). Nighttime pain due to peptic ulcer was found to be relieved by a long-acting anticholinergic, hexocylium. The conventional form was ineffective. By limiting the clinical study to a single symptom, nocturnal pain, Kasich (131) believes that the errors inherent in evaluating drug effects for peptic ulcer are reduced.

Reese and co-workers (132) evaluated a sustained-release capsule of belladonna alkaloids for anticholinergic activity by means of the salivary suppression test (133). The study was statistically well designed using a Latin square design to reduce the many variables that are frequently encountered in similar clinical evaluations. The results indicated that the salivary test was a reliable measure of salivary flow. A single dose of the nonsustained release form contained 0.2 mg. of belladonna alkaloids and its salivary suppression effect lasted less than six hours. The sustained-release form contained 0.8 mg. of belladonna alkaloids in a single dose and its effect persisted for about twelve hours. Quadrupling the dose only doubled the activity. The salivary test may be a good indicator of the drug's effect on the gastrointestinal tract. Bachrach (134) found that it was possible to establish a dose of three anticholinergics, including belladonna, which inhibited the salivary glands, but had no other subjective effects such as dysuria. The action of anticholinergics may vary in individuals and no one drug will consistently alter some gastrointestinal function in most patients. Bachrach, in his extensive review of anticholinergic drugs, states that the presence of dryness of the mouth which is frequently reported as a side effect of these drugs, does not assure the attainment of the desired gastrointestinal effect. Mushin and colleagues (133) have remarked that the subjective sensation of dryness frequently differs from the salivary index. Bachrach suggested that a more reliable method of obtaining prolonged action would be to incorporate the anticholinergic drug into a suppository for prolonged daytime effect. A sustained effect during the night could be obtained by the oral administration of a large dose in conventional form at bedtime, since the anticholinergic drug is apparently better tolerated during sleep. Also the reduced activity of the gastrointestinal tract permits maxiumum absorption of the drug. Bachrach questions the use of the term side effect as related to anticholinergics. Visual changes are reported as a side effect of a drug when it is given to a gastric ulcer patient, yet the ophthalmologist may laud the same drug because of its mydriatic response on the eye. Is it then a side effect?

THERAPEUTIC CONSIDERATIONS

Objections have been raised against the long-acting vitamin preparations which have been claimed to provide more efficient utilization, a continuous day-long supply of multivitamins, less waste, and similar unsubstantiated claims. Taking the total daily dose in several divided doses apparently has no effect on the excretion (and, therefore, presumably on the absorption) of riboflavin or ascorbic acid. However, thiamine and vitamin B_{12} are absorbed

in significantly greater amounts than when given as single doses. Contrary to claims made, none of the so-called "sustained-release" vitamin preparations on the market produced more efficient utilization of thiamine or riboflavin, or gave sustained urinary excretion of these vitamins. Since the utilization of riboflavin and ascorbic acid cannot be improved by giving the total dose in several divided doses, claims for improved utilization of these vitamins in sustained-release preparations are unjustified (135).

The advantages of continuous action of the oral long-acting dosage forms may be offset by the concomitant prolonged side effects of the drug. According to McHardy (136) this may create considerable concern for the anticholinergic-sensitive patient. Furthermore as the drug alters gastrointestinal motility and secretions it will probably alter its own absorption. It is McHardy's opinion that much can be gained through further study of prolonged-action drugs in their applicability to the management of disease.

The editors of *The Medical Letter* (137) voiced the difficulties involved in *in vitro* and *in vivo* testing of sustained-action drugs, but while questioning the value of some drugs in these forms, admit the therapeutic desirability of such products. They stated that the entire problem of the rate and the reliability of the disintegration of long-acting drugs deserved intensive investigation.

Dragstedt (138) said that a preparation which delays the "absorption of the active ingredient to any dependable degree, inevitably does so at the expense of some loss in precision of dosage." Wagner (88) reported that the amount of prednisolone available to human subjects in a twenty-four-hour period from two long-acting preparations was equal to that obtained from conventional tablets and that these results were at variance with the statements by Dragstedt.

Wilson (6) advises against the use of nitrites in oral prolonged-action forms for the treatment of angina of effort because the drugs may obscure the warning symptoms of pain and lead to overexertion with harmful results. The use of an ion exchange resin complex of an antihistamine and dihydrocodeine has received favorable comment. However, caution is suggested in the use of this preparation along with other morphine derivatives in patients with breathing difficulties because of the possibility of suppressing the respiratory center (139).

The marketed preparations do not offer an ideal release of the drug with a constant release per unit time, but instead exhibit a continuously decreasing release of drug with time (3, 8). Nelson differentiates between sustained release and prolonged action. His definition of an ideal sustained-release form is one in which the drug becomes available for absorption at a constant rate equal to the activity disappearance rate in the body after absorption. Prolonged-action drugs, on the other hand, are defined as those which "provide for replacement of drug at some rate which gives a measurable increase in the length of time activity." He states that sustained release, as he defines it, is difficult to achieve and currently available products are of prolonged-action type (8).

A satisfactory sustained-release product should be as independent as possible of the physiological conditions that exist in the gastrointestinal tract. Preparations which are dependent upon the pH, volume, and constituents of the gastric and intestinal secretions for the release of the drug may be less effective in conditions where the secretions or their constituents vary from the normal. Should stomach emptying time be delayed as may occur after a meal high in fat content, a preparation which requires the enzymatic activity of the intestinal secretion, or a neutral, or slightly alkaline pH for the release of some of the drug would have its activity delayed.

Since most drugs are absorbed to a greater extent in the upper part of the intestines than in the lower portion, the doses which are released after seven and eight hours should be higher than the fourth and fifth hours to compensate for the decreased absorption (3).

The development of prolonged-action dosage forms which will maintain constant and uniform therapeutic drug levels for eight to twelve or perhaps twenty-four hours requires more extensive work on the fate of the pure drug in the body following ingestion. It is evident that more emphasis must be placed on the development of methods for the quantitative determination of a compound in body fluids and in the excretions. Clarification is required of the mechanisms of detoxification and the biochemical pathways of metabolism and degradation. Closer collaboration between members of the various scientific disciplines as well as closer contact between clinicians and laboratory workers will result in drugs which are better tolerated, more active, and of longer and more uniform duration.

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Research Articles

Measurement of Hydrophile-Lipophile Balance of Surface-Active Agents

By ALEXANDER H. C. CHUN† and ALFRED N. MARTIN

An interfacial tension method was developed for the evaluation of hydrophile-lipophile balance (HLB) values of water-soluble surface-active agents. One-tenth per cent aqueous solutions of surfactant were overlayered with toluene and the interfacial tension measurements were made with a du Nouy tensiometer. A linear relationship resulted when the interfacial tension values were plotted against HLB values. This method does not apply to mixtures of surfactants and to natural emulsifiers. The HLB values are also related to H/L numbers.

WITH THE NUMEROUS surface-active agents now available, one of the major problems is to develop a method for the evaluation of surface-active agents so that they can be screened for different applications. Surface-active agents or surfactants, also referred to as amphipathic (1) and amphiphilic (2) agents, have been classified and identified by various methods. The most common classification is by ionic type such as nonionic, anionic, cationic, or ampholytic. Surfactants have also been classified both qualitatively and quantitatively according to a second method depending on the hydrophilic and lipophilic characteristics of the surfactant molecule. Methods such as hydrophile-lipophile balance (3), hydrophile/lipophile numbers (4), water number (5), and various designations used by manufacturers such as the Pluronic grid (6) and Igepal hydrophilic number (7) have been introduced in recent years.

The concept of balance of emulsifying agents was reported at an early date (8) but it was not until 1948 that a systematic classification of surfactants was devised (3, 9), called the hy-

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† Present address: Abbott Laboratories, Scientific Division, North Chicago, Ill.

drophile-lipophile balance or the HLB method. According to this system, the surfactants are assigned empirical numbers called HLB values. The lipophilic or "oil loving" compounds are assigned low numbers while the hydrophilic or "water loving" surfactants are assigned high numbers. From the HLB values one can determine the applications for various surfactants. Oils, waxes, and other emulsifiable substances are assigned "required HLB" values which should be matched by one or usually a mixture of surfactants to effect emulsification, solubilization, wetting, and other uses. The required HLB values (10) vary for different applications.

The experimental method for the determination of HLB values as first reported by Griffin (3) was time consuming. Later, equations (10) were given from which these values could be calculated for certain types of nonionic surfactants. The HLB values of nonionic surfactants containing ethylene oxide can be estimated by the cloud point (11) or temperature at which an aqueous solution of surfactant shows a sharp change in turbidity.

Greenwald, Brown, and Fineman (5) titrated a surfactant-solvent combination with water until a persistent turbidity was reached to determine the hydrophile-lipophile character of surfactants. The number of milliliters of water used to reach the end point was called the water number. This method is similar to the dilution ratio test used in the paint and varnish industry (12). The term "water number" was an unfortunate choice since it has previously been used in pharmacy by Casparis and Meyer (13) to designate the number of grams of water taken up by 100 Gm. of ointment base. Greenwald, Kice, Kenly, and Kelly (14) have recently reported on a method of determining hydrophile-lipophile balance involving the partition of the surface-active agent between iso-octane and water.

The term hydrophile/lipophile number or H/L number was introduced by Moore and Bell (4). The H/L number is defined as the number of ethylene oxide units multiplied by 100 and divided by the number of carbons in the lipophilic portion of the molecule. Of the methods, the hydrophile-lipophile balance or the HLB method appears to be the most promising for the classification of surfactants. However, there is as yet no rapid method for determining the hydrophile-lipophile values of surfactants. The object of this research is to devise a method for the evaluation of hydrophile-lipophile values and to relate the results to the composition and properties of surfactants.

EXPERIMENTAL

Materials.—Toluene, Baker analyzed grade, was tested by a modified gas-liquid chromatographic apparatus (15) and found to be of high purity. The surfactants were of commercial grade. Deionized distilled water was used throughout the study.

Instrumentation.—The du Nouy tensiometer, fitted with a platinum-iridium ring, circumference 6.008 cm. and an R/r ratio 53.8, was used for the interfacial tension measurements. The tensiometer was calibrated according to the instructions (16) provided with the instrument. The interfacial tension measurements were made after five minutes on a 0.1% aqueous solution of surfactant overlayered with toluene in a 50-ml. beaker at 25 ± 0.1°. Three measurements were made and averaged, and the correction factor of Zuidema and Waters (17) was applied to all of the measurements. The interfacial tension measurements made at five minutes gave sufficiently reproducible results to serve as a basis for the rapid test, therefore, no attempt was made to measure the interfacial tension values at equilibrium.

RESULTS AND DISCUSSION

Interfacial Tension *versus* HLB.—An interfacial tension study to determine HLB has not been considered previously presumably because of the difference observed in "pull" and "push" interfacial tensions and because the interfacial tensions of efficient emulsifier systems are close to zero. However, preliminary studies in our laboratories showed that the "pull" or "up" interfacial tensions for dilute solutions of surfactants against toluene was related to HLB.

A linear relationship resulted when the corrected interfacial tension (γ) was plotted against HLB, as shown in Fig. 1 and listed in Table I. The equation of the line determined by the method of least squares is given by the expression

$$\gamma = 45.7 - 2.36\,(\text{HLB}) \qquad \text{(Eq. 1)}$$

The HLB values can be calculated by determining interfacial tensions and placing these results in the expression

$$\text{HLB} = -(\gamma - 45.7)/2.36 \qquad \text{(Eq. 2)}$$

The equation was determined using the data from single batches of surfactants. Table I also lists other information on these surfactants.

Mixture of Surfactants.—The interfacial tensions of mixtures of surfactants of the polyoxyethylene type were determined to observe whether they followed the same relationship as the interfacial tension of the surfactants when used alone. The procedure in making the interfacial tension measurements was the same as outlined previously.

It was found that the interfacial tension of a mixture of surfactants was lower than expected. For example, the interfacial tension for a 50% mixture of Igepal CO-430 and Igepal CO-880 would be expected to be an average of the interfacial tensions of the two surfactants, or 15.4 dynes/cm., but it was found experimentally to be 7.1 dynes/cm. Figure 2 shows the relationship between the composition of the surfactant mixture and the interfacial tension. The greater the difference in HLB values

TABLE I.—PHYSICAL DATA FOR SURFACTANTS WITH KNOWN HLB VALUES

	Surfactant	Chemical Composition	Units Ethylene Oxide	HLB Value[a]	Interfacial Tension	H/L No.	Cloud Point 1%, °C.	Manufacturer[b]
1	Igepal CO-430	Nonylphenoxy polyoxyethylene	4	8.9	26.1	26.7	...	1
2	Igepal CO-530	Nonylphenoxy polyoxyethylene	6	10.9	20.4	40	0	1
3	Igepal CO-630	Nonylphenoxy polyoxyethylene	9.5	13.1	14.5	63.3	54	1
4	Igepal CO-710	Nonylphenoxy polyoxyethylene	10.5	13.5	12.8	70	72	1
5	Igepal CO-730	Nonylphenoxy polyoxyethylene	15	15	9.7	100	97	1
6	Igepal CO-850	Nonylphenoxy polyoxyethylene	20	16	7.6	133	>100	1
7	Igepal CO-880	Nonylphenoxy polyoxyethylene	30	17.1	4.7	200	>100	1
8	Renex 648	Nonylphenoxy polyoxyethylene	5	10	23.3	33.3	...	2
9	Renex 697	Nonylphenoxy polyoxyethylene	6	10.9	20.4	40	0	2
10	Renex 688	Nonylphenoxy polyoxyethylene	8	12.3	18	53.3	30	2
11	Renex 678	Nonylphenoxy polyoxyethylene	15	15	10.9	100	99	2
12	Renex 698	Nonylphenoxy polyoxyethylene	9.25	13	14.7	61.6	54	2
13	Renex 690	Nonylphenoxy polyoxyethylene	10	13.3	13.8	66.7	66	2
14	Renex 35	Polyoxyethylene tridecyl alcohol	12	14.5	11.2	120	82	2
15	Renex 31	Polyoxyethylene tridecyl alcohol	15	16.0	7.5	150	>100	2
16	Renex 30	Polyoxyethylene tridecyl alcohol	12	15	11.4	120	84	2
17	Renex 36	Polyoxyethylene tridecyl alcohol	6	11.4	17.9	60	...	2
18	Tween 40	Polyoxyethylene sorbitan monopalmitate	20	15.6	7.8	...	...	2
19	Tween 60	Polyoxyethylene sorbitan monostearate	20	14.9	10	...	...	2
20	Tween 21	Polyoxyethylene sorbitan monolaurate	4	13.3	14.4	...	...	2
21	Tween 81	Polyoxyethylene sorbitan monooleate	5	10	20.1	...	...	2
22	Tween 80	Polyoxyethylene sorbitan monooleate	20	15	9.1	...	...	2
23	Tween 20	Polyoxyethylene sorbitan monolaurate	20	16.7	6.9	...	...	2
24	Triton X-100	*t*-Octyl polyoxyethylene	9.5	13.4	15.0	69.3	65	3
25	Triton X-102	*t*-Octyl polyoxyethylene	12.5	14.6	11.1	87.9	88	3
26	OPE 16	*t*-Octyl polyoxyethylene	16	15.5	9.1	114	>100	3
27	OPE 20	*t*-Octyl polyoxyethylene	20	16.2	7.6	143	>100	3
28	OPE 30	*t*-Octyl polyoxyethylene	30	17.3	5.8	214	>100	3
29	OPE 40	*t*-Octyl polyoxyethylene	40	17.9	4.8	286	>100	3
30	Brij 30	Polyoxyethylene lauryl ether	4	9.5	23.4	33	...	2
31	Brij 35	Polyoxyethylene lauryl ether	23	16.9	6.3	192	...	2
32	Myrj 49	Polyoxyethylene monostearate	...	15	12	...	...	2
33	Myrj 51	Polyoxyethylene monostearate	...	16	8.8	...	...	2
34	Myrj 52	Polyoxyethylene monostearate	40	16.9	7.9	222	...	2
35	Myrj 53	Polyoxyethylene monostearate	...	17.9	7.4	...	...	2
36	Igepal CA-630	Iso-octyl phenoxypolyoxyethylene	...	12.8	15.2	...	...	1
37	Emulphor ON-870	Polyoxyethylene fatty alcohol	...	15.4	11.4	...	...	1
38	OPE 1	*t*-Octyl phenoxy polyoxyethylene	1	3.5	...	7.14	...	3
39	OPE 3	*t*-Octyl phenoxy polyoxyethylene	3	7.8	...	21.4	...	3
40	Triton X-45	*t*-Octyl phenoxy polyoxyethylene	5	10.3	...	35.7	...	3
41	Triton X-114	*t*-Octyl phenoxy polyoxyethylene	7.5	12.3	...	57.3	21	3
42	Atlas G-3920	Polyoxyethylene oleyl alcohol	20	15.4	...	112	...	2
43	Myrj 45	Polyoxyethylene monostearate	8	11.1	...	44	...	2
44	Renex 650	Nonylphenoxy polyoxyethylene	30	17.1	...	200	>100	2

[a] HLB values calculated or as reported (10).

[b] The following companies supplied their surfactants for this part of the study: 1. Antara Chemicals, a Sales Division of General Aniline and Film Corp., 435 Hudson St., New York 14, N. Y. 2. Rohm and Haas Co., Washington Square, Philadelphia 5, Pa. 3. Atlas Powder Co., Wilmington 99, Delaware.

of the surfactants (ΔHLB) in the mixture the greater was the deviation in the experimental interfacial tension (γ) from the expected (γ'). This phenomenon is illustrated in Fig. 3.

These results correspond to the cloud point data for mixtures of surfactants reported by Maclay (18). A higher cloud point value for mixtures of surfactants resulted when two surfactants with a widely different distribution of ethylene oxide were mixed. According to Maclay, this phenomenon may be due to the fact that the more hydrophilic surfactant solubilized the less hydrophilic surfactant and accordingly contributed more to the property being measured. The deviation of interfacial tension for the mixtures of surfactants might be explained by the same mechanism.

Since the interfacial tension of mixtures of surfactants deviated from the expected values, emulsions were prepared to observe whether the HLB values for the mixture of surfactants determined by the interfacial tension method corresponded to the HLB values determined by the emulsion method.

A mixture of 1% each of stearic acid and light

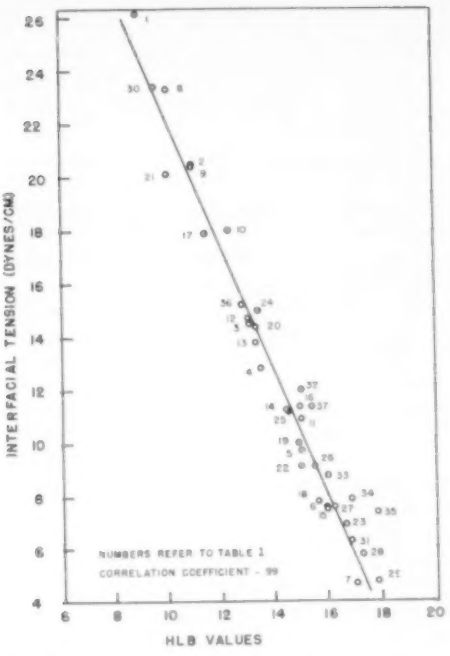

Fig. 1.—Interfacial tension *vs.* HLB values of surfactants with known HLB values.

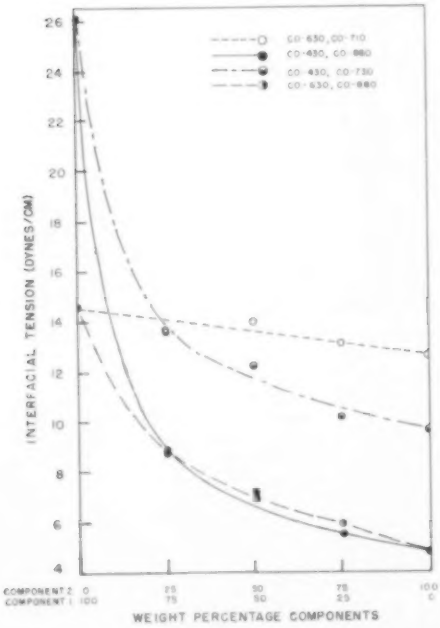

Fig. 2.—Interfacial tension values of mixtures of surfactants.

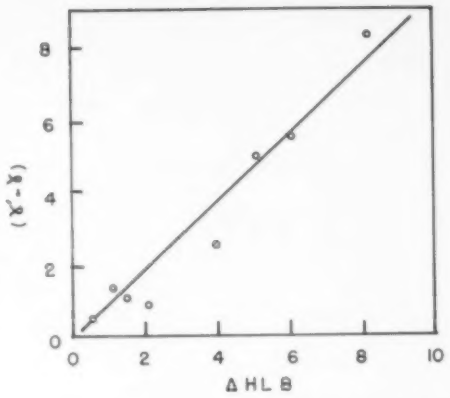

Fig. 3.—The deviation of interfacial tension values of 50% mixtures of surfactants.

liquid petrolatum U. S. P., was used as the internal phase. The procedure in making the emulsions was similar to that used by Chun, Joslin, and Martin (19), except that the emulsions were homogenized once through a quart-size hand homogenizer while the mixture was at about 60°. The emulsions were evaluated by a shelf aging method and a centrifugal method. The best emulsions found by both methods were in agreement, but the HLB of the emulsions did not correspond with the HLB of the mixtures of surfactants as determined by the interfacial tension method; therefore, the interfacial tension method cannot be used to predict the HLB values of mixtures of surfactants of widely different HLB. However, it is possible that the decrease in interfacial tension may have some bearing on complexation between the surfactants, which in turn could serve as a measure of increased stability of emulsions. This may provide a clue to the stabilization of emulsions, the rule being to choose agents of widely different hydrophile-lipophile character (HLB) of the proper chemical type in order to form a strong interfacial complex and consequently a stable emulsion. This interesting possibility must be tested in a later study.

The interfacial tension method was used to determine the HLB values of the natural products such as acacia, tragacanth, and gelatin. The results did not correspond to the values (19) determined by the emulsion method, but the results gave indication of the relative hydrophile and lipophile character of these agents. A possible explanation is that the natural emulsifiers do not form emulsions predominately by the lowering of interfacial tension as do the synthetic agents. Hence, this method which was used for the synthetic agents cannot be used to determine the HLB of the natural emulsifiers.

H/L Numbers *versus* HLB values.—Moore and Bell (4) introduced the term hydrophile/lipophile numbers or H/L numbers for surfactants. The H/L numbers for various nonionic surfactants were calculated according to equation

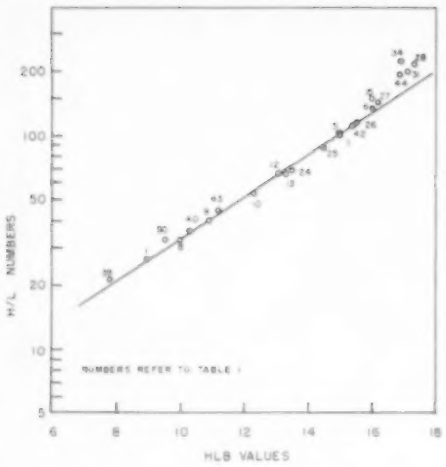

Fig. 4.—The relationship between H/L numbers and HLB values.

$$H/L = \frac{\text{number of oxyethylene units} \times 100}{\text{number of carbons in lipophile}} \qquad \text{(Eq. 3)}$$

Some of these are listed in Table I. A linear relationship resulted when the H/L numbers were plotted as ordinates and the HLB values as abscissas on semilog paper for 23 surfactants as shown in Fig. 4. The H/L numbers and HLB values exhibited a good relationship, except for the surfactants with a distribution of greater than 20 ethylene oxide units, where the H/L number deviated from the line as shown in Fig. 4. The slope of the line was calculated to be 0.0971 and the equation of the line as

$$\log (H/L) = 0.0971 (HLB) + 0.544 \qquad \text{(Eq. 4)}$$

As there was a good correspondence between the H/L numbers and the HLB values, the H/L numbers should serve as a method of estimating HLB values, and vice versa, for nonionic surfactants containing ethylene oxide as the hydrophilic portion of the molecule. Since interfacial tension was related to HLB, and HLB was related to H/L, the interfacial tension values could also be used to predict H/L numbers.

SUMMARY

1. A rapid interfacial tension method for the determination of hydrophile-lipophile balance values was developed for a number of water-soluble surfactants.

2. The interfacial tensions of mixtures of surfactants of the polyoxyethylene type was intermediate between the values for single surfactants. The results for the mixture of surfactants did not agree with the HLB values obtained by the emulsion method.

3. The interfacial tension method for the determination of HLB for natural emulsifiers did not correspond with the results from the emulsion method.

4. The H/L number and HLB values of a number of surfactants are related exponentially, but the relationship deviated for compounds containing greater than 20 units of ethylene oxide.

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Alfalfa Extracts Which Stimulate Growth of *Neurospora sitophila* I

Preparation and Activation of Crude Extracts

By JORDAN G. LEE, JOSEPH A. LIUZZO, and ARTHUR F. NOVAK

Extracts of alfalfa leaf meal may increase or decrease the mycelial weight of *Neurospora sitophila* in the standard Difco pyridoxine assay. The effect obtained was found to be dependent upon the solvent employed or the subsequent treatment of the extract. Water extracts were inactive or inhibitory. Extracts with 0.1 *N* or stronger hydrochloric acid were active. Increasing the temperature or time of extraction favored activation only slightly. The inactive water extracts could be activated by the addition of hydrochloric acid or by the extraction of inhibitory material with diethyl ether or dimethylformamide.

IT HAS BEEN shown (1) that 0.1 *N* hydrochloric acid extracts of alfalfa leaf meal increase the mycelial weight of *Neurospora sitophila* when added to a medium previously considered complete for growth of the mold and still widely used in a biological assay for pyridoxine. A concentrate retaining this property has been prepared by Novak, Jonnard, and Liuzzo (2).

The evaluation of the desirability of procedures to be used in isolating the factor, or factors, responsible for this increase required the definition of assay deviations, qualitative and quantitative, which were to be considered significant. A means of assessing the efficiency of separation and concentration achieved by these procedures was also considered desirable.

PRELIMINARY STUDIES

A series of 25 assay flasks containing no added alfalfa fractions were prepared with media from three commercial lots. These were treated as control flasks would have been under standard assay conditions. Least significant differences from the mean, at a confidence level of 1%, were calculated for each series. The greatest LSD was 1.0 mg., which was 2.25% of the mean value. A deviation in an assay of more than 5% from the control value was then arbitrarily considered as being significant, provided it could be duplicated in a repeated assay.

A second problem in assay evaluation was the occurrence of sporulating, heavily pigmented cultures when certain alfalfa fractions or pure compounds were present in the assay medium; occasionally, this also occurred in control flasks. The mycelial weights from such assay flasks were very high. This pattern of development did not occur with crude extracts, although such extracts also produced increased mycelial weight. Barton-Wright (3) stated that increased variability was found where sporulation occurred. He advocated the addition of Zn^{++} to suppress the phenomenon. Under such circumstances, a chelating agent without direct effect upon mycelium production might alter the developmental pattern to indirectly produce weight increase. The highly active alfalfa extracts always produced very rapid growth without such heavy, pigmented areas. It was, therefore, decided that only fractions producing both increased mycelial weight and the same qualitative growth response as the active extracts would be considered as containing stimulatory material.

The problem of extraction efficiency was next studied. Assay values were obtained for extracts prepared with distilled water or dilute hydrochloric acid as solvent. Two extraction temperatures and various heating times were employed with each solvent.

The results, recorded in Table I, indicate that temperature and time of heating were minor, though significant, factors in producing active extracts. They also show that the acidity of the solvent was the major factor. Further, the results with preparations 14 through 17 indicate that this H^+ effect lies not in extraction from the alfalfa, but is an effect upon the water-solubles themselves.

Qualitative differences were observed between cultures containing water extracts and activated extracts. Where an untreated water extract was present, growth proceeded at an accelerating pace from a small number of centers; these might not appear for twenty-four hours or more. The active, acid-treated extracts, on the contrary, displayed extremely rapid initial growth which was initiated uniformly throughout the flask. These two patterns are consistent with the hypothesis that the activation process consists of removal of inhibitory materials.

In an earlier paper (2), evidence had been presented that diethyl ether removed inhibitory material from extracts of alfalfa. Accordingly, this procedure was applied to an inactive water extract. The ether solubles were found to be inhibitory. The ether-extracted water extract, previously inactive, was now active. See the results obtained with preparations No. 18, 21, and 22 in Table I. An analogous result was obtained when the inactive water extract was evaporated to dryness *in vacuo*, then extracted with dimethylformamide. The dimethylformamide-soluble material possessed inhibitory powers. The residue had become active. It should be emphasized that both of these procedures produced insoluble solids which were re-

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Table I.—Activity of Extracts under Varied Conditions of Extraction and Treatment

Prepn. No.	Extraction Procedures	Assay Value[a]
	Water Extn. at	
1	25° for 18 hr.	92
2	121° for 10 min.	102
3	121° for 30 min.	100
4	121° for 60 min.	98
	0.1 *N* HCl Extn.	
5	25° for 18 hr.	135
6	121° for 10 min.	140
7	121° for 30 min.	144
8	121° for 60 min.	147
	Extn. at 121° for 30 min.	
9	Water	93
10	0.01 *N* HCl	103
11	0.1 *N* HCl	137
12	0.4 *N* HCl	140
13	1.0 *N* HCl	144
	Treatment of Extracts	
14	Water ext. prepd. at 25°	82
15	No. 14 heated 15 min. at 121°	80
16	No. 14 made 0.1 *N* HCl, then heated 15 min. at 121°	133
17	No. 14 made 1.0 *N* HCl, then heated 15 min. at 121°	164
18	Water ext. prepd. at 121° for 15 min.	93
19	DMF solubles of No. 18	76
20	DMF insolubles of No. 18	141
21	Material from No. 18 sol. in 50% ethanol and in diethyl ether	55
22	Material from No. 18 sol. in 50% ethanol and insol. in diethyl ether	148

[a] Control = 100.

moved and did not appear in the final soluble fractions.

It was evident that the apparent activity of alfalfa extracts was governed by the presence of both inhibitory and stimulatory material. Further, the balance, presence, or availability of these was governed by the nature of the treatment of the extract. The objective of formulating a unit of activity to be used in assessing the efficiency of extraction or separation procedures was, therefore, abandoned. No attempt has been made to estimate the recovery of, or contributions of the various fractions to, the original activity.

The activation process may be one of making available, or of producing, stimulatory substances. It may also be a question of removal of inhibitory material. A definite mechanism cannot be offered until the compounds involved have been isolated. However, the inhibited pattern of growth displayed when the crude water extract was present, plus the inhibitory property of the diethyl ether and dimethylformamide solubles, suggest that the mechanism of activation is primarily the removal of inhibitory material. Whatever the cause, extreme care must be exercised in applying this pyridoxine assay to varied types of extracts, for the mycelium production may be governed by treatment of the extract and not solely by pyridoxine content.

EXPERIMENTAL METHODS

Microbiological Assay.—The assay method was as described by Novak, Jonnard, and Liuzzo (2). All assays were conducted with that amount of preparation equivalent to 1.0 Gm. of alfalfa leaf meal.

Assay values are relative values with the control assigned a value of 100.

Extraction Procedures.—Commercial alfalfa leaf meal was extracted with solvent in a 5 ml. per Gm. proportion. Extracts prepared at room temperature were stirred thoroughly to prevent caking, then allowed to sit for eighteen hours before filtering. Heated extracts were autoclaved at 121° for the indicated times, then allowed to sit overnight at room temperature before filtering. Initial solids removal consisted of filtration through heavy cotton cloth sacks followed by manual expression of fluid.

In preparing extracts 1 through 13 the pH was adjusted by addition of sodium hydroxide or hydrochloric acid, the solutions were centrifuged until clear, and the volume brought to a 4 ml. per Gm. alfalfa proportion.

Where subsequent treatment followed, as in preparations 14 through 22, the crude filtrate was centrifuged until clear, then divided into the requisite number of equal-volume aliquots before proceeding. In each case, the preparation was centrifuged after final pH adjustment and brought to a 4 ml. per Gm. alfalfa proportion.

Preparations No. 19 and 20 were prepared by evaporating an aliquot of No. 18 to dryness *in vacuo* below 40° in a rotating evaporator and extracting by shaking the solids with dimethylformamide until the extracts were colorless. The residue was dissolved in water and again taken to dryness at 40° *in vacuo*. These solids were then redissolved in water for pH adjustment as previously described. The dimethylformamide extract was evaporated to dryness *in vacuo*, then treated just as the residue was treated.

Preparations No. 21 and 22 were prepared from No. 18 as described by Novak, Jonnard, and Liuzzo (2).

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Alfalfa Extracts Which Stimulate Growth of *Neurospora sitophila* II

Isolation, Characterization, and Multiple Nature of Stimulatory Compounds

By JORDAN G. LEE, JOSEPH A. LIUZZO, and ARTHUR F. NOVAK

Acidic extracts of alfalfa leaf meal increase the mycelial weight of *Neurospora sitophila* in the Difco pyridoxine assay method. This stimulation is caused by the presence of isoleucine, cystine, and at least two unidentified factors in such extracts. A method for the isolation of these two unidentified factors is presented.

IT HAS BEEN shown (1) that acidic extracts of alfalfa leaf meal increase the mycelial weight of *Neurospora sitophila* when added to a medium considered complete for the biological assay of pyridoxine. A concentrate retaining this property has been prepared by Novak, Jonnard, and Liuzzo (2).

The reported properties and unpublished data indicated that more than one stimulatory substance was present in extracts of alfalfa. Further, it seemed probable that these materials were cations in neutral or acid solution. Fractionation of crude extract on a Dowex 50 (H^+) column by the method of Mueller, *et al.* (3), was, therefore, attempted (preparation scheme 1).

PRELIMINARY STUDIES

The material which passed through the column with distilled water as solvent was inactive. The next solvent, 0.8 *N* hydrochloric acid in 55% ethanol, eluted the acidic and neutral amino acids. One of the fractions eluted by this solvent was active, assay value 112. Paper chromatograms indicated this active fraction contained isoleucine. Normal hydrochloric acid was then passed through the column to elute inorganic material. Assay showed that this material was inactive. Finally, 6 *N* hydrochloric acid was passed through the column to remove the basic amino acids. Fractions 2, 3, and 4 of the 6 *N* eluate gave assay values of 141, 118, and 134, respectively. Three ninhydrin reactive compounds with R_f values below 0.3 were present in all three stimulatory fractions. Two of these were fluorescent, on paper, under ultraviolet light.

Fractions 2 and 3 of the 6 *N* eluates were then individually subfractionated on preparative paper chromatograms into four zones. These were individually assayed. Assay values, in numerical order, obtained for zones 1 through 4 of fraction 2 were 106, 110, 113, and 100. The corresponding values for fraction 3 were 103, 113, 110, and 105. The most active zones were ninhydrin-reactive, fluorescent bands. On paper chromatograms, these moved between known samples of L-lysine and L-cystine. They did not behave chromatographically as did any of the common amino acids.

Amino acids, selected on the basis of these results, were individually assayed at a level of 5 μM per assay flask. Values found were: L-leucine, 95; L-isoleucine, 112; L-cystine, 110; L-arginine, 100; L-lysine, 97; D,L-histidine, 99; L-aspartic acid, 99; D,L-ornithine, 98. This stimulatory effect of isoleucine and of cystine has been repeatedly observed.

These results indicated that the stimulatory property of alfalfa extracts resided in at least three components, isoleucine and two unidentified materials. Although stimulatory, cystine was not definitely shown to be in any of these active alfalfa fractions. It has been shown chromatographically to be present in crude acidic extracts, however.

A solvent fractionation approach, which had earlier indicated that the stimulatory properties resided in more than one substance, was now repeated (preparation scheme 2). Here, the dried solubles of an active 0.1 *N* extract were successively extracted with acetone, butanol, 2-propanol, ethanol, 95% ethanol, methanol, and water. Only the butanol and the water fractions increased mycelial weight above the control value. Their assay values were 108 and 127, respectively.

Paper chromatograms of the butanol solubles showed isoleucine was present. The isoleucine band was eluted from a preparative chromatogram of this fraction and assayed. An assay value of 118 was obtained. Cystine was not found.

Paper chromatograms of the final water extract had no ninhydrin-reactive zones with the quantities applied, though fluorescent zones were present. The acid hydrolyzate of this fraction gave at least six ninhydrin-reactive spots. Two of these were also fluorescent. Neither cystine nor isoleucine was present. The total hydrolyzate gave an assay value of 128. The combined eluates from the two fluorescent, ninhydrin-reactive zones gave an assay value of 128.

These results led to the same conclusions as did the results of scheme 1. They further indicated that the unidentified stimulatory materials were present in bound form, from which they were liberated by hydrolysis.

Accordingly, a hydrolytic step and a carbohydrate removal step were included in the third preparation scheme prior to passage through the Dowex 50 (H^+) column. Acidic alcohol was not used as an eluant.

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Isoleucine emerged in fractions 32–35 of the *N* hydrochloric acid eluate. No residual solute was observed in fractions 36–60. Therefore, use of 2 *N* hydrochloric acid as eluant was begun. Almost immediately, a weakly ninhydrin-reactive, fluorescent, white, deliquescent solid appeared in traces in fractions 63–65. This appeared to be chromatographically identical with the faster moving, fluorescent zone previously found stimulatory.

Fractions 76–80 contained traces of a strongly ninhydrin-reactive, fluorescent, white, deliquescent solid solute. This appeared to be chromatographically identical with the previously observed slower-moving, fluorescent, stimulatory zone.

Assay values of 122, 117, 110 were obtained for fractions 63, 65, and pooled fraction 76–80, respectively. The combination of pooled fraction 76–80 with fraction 63 gave an increased assay value of 133; with fraction 65, a value of 128.

These results indicate that the stimulatory effect of crude alfalfa extracts is the result of multiple influences whose possible interaction remains unknown. Isoleucine is definitely one of these stimulatory materials. Cystine is very probably a factor in the activity of crude extracts of alfalfa. At least two unidentified stimulatory substances are also present, and a method for their isolation has been presented. These seem to occur in a bound form, from which they may be liberated by acid hydrolysis.

EXPERIMENTAL METHODS

Crude Extract.—Commercial alfalfa leaf meal was extracted with 0.1 *N* hydrochloric acid in a 5 ml. per Gm. proportion by autoclaving at 121° for fifteen minutes. After cooling to room temperature, the mixture was filtered and the residue washed with water. The filtrate and washings were combined, evaporated to dryness on a steam bath, and redissolved in distilled water. Insoluble material was removed by centrifugation. The final volume was adjusted so that 1 ml. was equivalent to 1 Gm. of alfalfa leaf meal.

Microbiological Assay.—The assay method was as described by Novak, Jonnard, and Liuzzo (2). Unless otherwise stated, all assays were conducted with that amount of preparation equivalent to 1 Gm. alfalfa meal.

Activities are expressed as relative values with the control flask assigned a value of 100.

Paper Chromatography.—For routine qualitative purposes, samples of 2 and 10 μL. were applied to Whatman No. 1 paper, and descending chromatograms were made using butanol:acetic acid:water (4:1:1) as solvent. These were run for sixteen to eighteen hours at room temperature. The air-dried chromatograms were examined with near and far ultraviolet, dipped in 0.1% ninhydrin in acetone, air-dried, and heated at 70–80° for ten minutes.

Preparative chromatograms each contained solute equivalent to 1 Gm. alfalfa leaf meal streaked on a 46 × 57 cm. sheet of Whatman No. 1 paper. The bands were marked under ultraviolet light and by use of guide strips treated with ninhydrin. The marked zones were cut out, individually eluted with 0.01 *N* hydrochloric acid, evaporated to dryness on the steam bath, and redissolved in distilled water so that 1 ml. was the equivalent of 1 Gm. alfalfa leaf meal.

Preparation 1.—Ten milliliters of crude extract was applied to a 1.5 × 60 cm. column of 200–400 mesh Dowex 50-X8 (H^+) resin and washed through with 100 ml. distilled water. Fifteen 50-ml. fractions were then collected using 0.8 *N* hydrochloric acid in 55% ethanol as eluant. The column was washed with 250 ml. *N* hydrochloric acid. This effluent was collected as one fraction. Eight 50-ml. fractions were now collected as 6 *N* hydrochloric acid passed through the column. All fractions were individually evaporated to dryness over steam and taken up in 10 ml. distilled water.

Preparation chromatograms were prepared with 1-ml. portions of fractions 2 and 3 of the 6 *N* eluate. Four zones were cut out and eluted from each sheet.

Zone 1 was the area from the origin to the slowest moving, fluorescent, ninhydrin-positive band.

Zone 2 comprised only this fluorescent, ninhydrin-positive band of low R_f.

Zone 3 comprised the faster moving fluorescent, weakly ninhydrin-positive area immediately above zone 2.

Zone 4 was the weakly ninhydrin-positive area just above zone 3.

Preparation 2.—Fifty grams of alfalfa leaf meal was extracted as for preparation of crude extract. The filtrate and washings were combined, concentrated *in vacuo* below 40°, and two volumes of 95% ethanol were added. The mass was thoroughly mixed, and the mixture allowed to stand overnight in the refrigerator. The precipitate was removed by filtration and discarded. The filtrate was concentrated *in vacuo* to a thick syrup, mixed with 50 Gm. of thoroughly washed and air-dried paper pulp, and the mix air-dried. The gummy mass was then extracted with acetone for ten hours in a Soxhlet extractor.

The acetone-extracted pulp was then placed in a Waring Blendor and extracted successively with butanol, absolute ethanol, and 95% ethanol. Five 400-ml. portions of each solvent were used, beating time was ten minutes for each portion. Solvent was removed each time by suction filtration.

The air-dried pulp residue was next extracted for forty-eight hours with absolute methanol in a Soxhlet extractor.

The pulp residue was next extracted with two 250-ml. portions of distilled water using a blendor as before.

All extracts were individually evaporated to dryness *in vacuo*, and each was redissolved in 50 ml. distilled water.

Forty milliliters of the final water extract was mixed with 40 ml. 37% hydrochloric acid, boiled under reflux for sixteen hours, evaporated to dryness on the steam bath, taken up in 40 ml. distilled water, and centrifuged.

A preparative chromatogram of 1 ml. of the clear hydrolyzate was made. The fluorescent bands, which appeared to be the same as zones 2 and 3 of preparation 1, were cut out and eluted as one zone because separation was not as definitive as before.

Preparation 3.—Ten milliliters of 37% hydrochloric acid was added to 10 ml. of crude extract, the mixture was boiled under reflux for sixteen hours, evaporated to dryness over steam, extracted by boiling with three successive 25-ml. portions of pyridine, taken up with 25 ml. of water, and again evaporated to dryness. The pyridine-free residue

was taken up in 50 ml. distilled water and centrifuged.

The clear supernatant was now washed through a 1.5 × 60 cm. column of 200–400 mesh Dowex 50-X 8 (H^+) with 250 ml. distilled water. Normal hydrochloric acid was now introduced, and sixty 25-ml. fractions were collected. Hydrochloric acid 2 *N*, was then passed through the column, and twenty-one 25-ml. fractions were collected. Finally, 200 ml. of 6*N* hydrochloric acid was passed through the column and collected as one fraction.

Fractions 76–80 were pooled and treated as one fraction. All other fractions were individually evaporated to dryness, and each was redissolved in 10 ml. distilled water.

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Alfalfa Extracts Which Stimulate Growth of *Neurospora sitophila* III

Effect on Weight Gain of Thyroid-Stressed Albino Rats

By JORDAN G. LEE, ARTHUR F. NOVAK, and JOSEPH A. LIUZZO

A concentrate prepared from alfalfa leaf meal which retains the property of stimulating growth of *Neurospora sitophila* in the standard Difco pyridoxine assay failed to increase weight gain in the albino rat when added to a purified diet. When the test animals were subjected to stress by feeding desiccated thyroid, the soluble concentrate did increase weight gain. It was as effective as an equivalent quantity of alfalfa leaf meal.

THE benificient nutritional qualities of alfalfa leaf meal are well known. Thus, the preparation by Novak, Jonnard, and Liuzzo (1) of an alfalfa concentrate which was highly effective in stimulating growth of *Neurospora sitophila* raised the question as to whether this preparation would also affect the growth of laboratory animals. Liuzzo, *et al.* (2), have reported a growth response of the chick as a result of including the intermediate fraction B of the scheme of Novak, *et al.* (1), in a practical ration.

PRELIMINARY STUDIES

In testing the effect of the concentrate upon growth of the albino rat, comparisons were made using weanling litter-mates paired for weight and sex. Introduction of the concentrate of Novak, *et al.* (1), into the basal purified diet at a level equivalent to 5% alfalfa leaf meal did not alter the thirty-day weight gain of six litter-mate pairs. This was not unexpected, as this purified basal ration has given good growth and reproduction through four generations of our rat colony.

Alfalfa and alfalfa leaf meal are among the many crude materials (3, 4) which have been shown to improve growth and livability of the thyroid-fed albino rat. The basal ration was, therefore, altered by the inclusion of U. S. P. thyroid as 0.1% of the ration and by doubling the content of B vitamins. Animals fed this basal diet had a depressed growth rate but only occasionally displayed any sign of severe stress.

The weight gains obtained using this basal supplemented with alfalfa leaf meal or the test concentrate are shown in Table I. It is apparent that both increased weight significantly when compared to the thyroid-containing basal ration. The concentrate was as effective as the meal. A sex difference is also discernible, with males giving the greater response to the concentrate. It is probable that their total stress, growth plus fed thyroid, was greater than that of the females.

These results are not strictly comparable with those in the literature because of the lower level of thyroid in our ration. Still, Overby and Fredrickson (3) have reported a protective effect with 5% alfalfa leaf meal and of forage juice fed at the same dry matter level. Ershoff, *et al.* (4), did not find evidence of protective action with 5% dried alfalfa juice or 5% water-soluble extract of alfalfa; pre-

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TABLE I.—EFFECT OF ALFALFA AND ALFALFA CONCENTRATE ON THIRTY-DAY WEIGHT GAINS OF THYROID-STRESSED WEANLING ALBINO RATS

No. of Pairs	Sex	Mean Difference, Gm.	s	t
Basal *vs.* Basal + 5% ALM[a]				
11	M	+11.5	15.82	2.40[b]
13	F	+ 6.5	9.62	2.42[b]
Basal *vs.* Basal + Concentrate[a]				
13	M	+11.1	8.97	4.45[c]
15	F	+ 4.6	7.98	2.20[b]
Basal + Concentrate[a] *vs.* Basal + 5% ALM				
7	M	− 3.9	5.70	2.25
9	F	+ 2.7	7.98	0.57

[a] Level equivalent to 5% ALM (alfalfa leaf meal). [b] $P < 5\%$. [c] $P < 1\%$.

parative methods were not given. They did find that the inclusion of either 20% alfalfa meal or of 15% of the residue left after water extraction of alfalfa meal increased the survival time of their extremely hyperthyroid animals. It is evident from these papers, and others cited by them, that protective action is associated with nitrogen-rich source material and that solubilization of the protective material has been found difficult. It is, therefore, of interest and importance that we are dealing here with a water-soluble concentrate which contains a number of ninhydrin-reactive substances.

The preceding paper in this series (5) describes the preparation, from alfalfa leaf meal of ninhydrin-reactive substances which are stimulatory for *Neurospora*. The relationship of these specific compounds to growth of thyroid-fed rats remains to be investigated. It should be emphasized, however, that the alfalfa concentrate of Novak, *et al.* (1), has now been found to contain, in soluble form, material promoting growth in the thyroid-stressed rat. In addition, the fraction B of this preparative scheme has been found to increase chick growth (2) under practical conditions.

EXPERIMENTAL METHODS

Rations Used.—The basal ration contained 20% vitamin-test casein (Nutritional Biochemicals), 5% corn oil fortified with fat-soluble vitamins, 3% dextrose fortified with B vitamins, 2% alphacel, 4% Hubbel, Mendel, and Wakeman salt mixture, and 66% sucrose. The fortified corn oil and fortified dextrose were prepared as described by Lee, Dupuy, and Rolfs (6).

This ration was modified to produce stress by including 0.1% U. S. P. desiccated thyroid and increasing the B complex-fortified dextrose to 6%. The sucrose content was reduced by an equal amount.

The alfalfa concentrate was prepared according to Novak, Jonnard, and Liuzzo (1) so that 1.0 Gm. dry matter was the equivalent of 1.0 Kg. alfalfa leaf meal. It was introduced into the ration at a level equivalent to 5% alfalfa leaf meal. The alfalfa leaf meal was incorporated at a 5% level in place of sucrose.

Test Conditions.—The albino rats used were from our local closed colony maintained at constant temperature. Three days prior to weaning, the stock ration was replaced by the basal ration. Two days after weaning, one pair matched for sex and weight was selected from a litter and transferred to individual cages. Water and the rations to be compared were given *ad libitum*. Body weight and food intake were recorded daily over the thirty-day test period. Thus, all comparisons were between paired litter mates, and each pair of Table I is from a different litter.

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Relation of pH to Preservative Effectiveness I

Acid Media

By DURWARD N. ENTREKIN

Tripticase soy broth adjusted to pH 2, 3, 4, 5, and 6 with McIlvane buffer was used as media for this study. Microorganisms for the inoculum were obtained from a soil sample which contained Gram-positive and Gram-negative rods, cocci, yeast, and fungi. Some of the preservative materials have been previously investigated on a limited scale and the *p*-hydroxybenzoates were used as a basis for comparison. The preservative materials were used in three concentrations spanning their theoretical effective range. A record was made of visible growth, bacteriostatic or bactericidal effect at each concentration and pH value and these were compared to control tubes. Effectiveness of some of the better known preservatives was confirmed while some of the newer materials fell into the effective class and others seeming to be ineffective.

THE PRESERVATION of medicinal and food products has been a continuing struggle for man. Salts and various spices have been used since time immemorial to preserve materials but frequently these strong tasting and highly volatile substances only covered up the fact that the product was spoiled. Most of the chemicals investigated, and found effective, in the early twentieth century are still in common usage today, indicating a serious lack of creative effort to produce preservative substances significantly better than those used over the past half century. It is the opinion of the author that the area of pH as it relates to antimicrobial activity deserves more attention than has been given it.

Taub, *et al.* (1), stated that since most pharmaceutical products are prepared at pH's below 7, the selection of pH 7 for evaluating the preservative action of bactericidal agents is unduly limiting and may result in conclusions which may not truly reflect the usefulness of this material. The author shares this opinion and feels that it should be expanded to state that any one pH level being used to test the effectiveness of a preservative material is likely to give a limited or incorrect value to the chemical being tested. This opinion was formed after observing that most of the recently published preservative studies were limited to a single pH value.

It has been stated as far back as 1926 that antiseptics are most effective at some definite pH which is different for each group of antiseptic materials (2). Barr and Tice (3), using pH levels from 2 to 8, determined that pH alone was not sufficient to preserve solutions of sorbitol. LeMar and White (4) found that the greater the hydrogen ion concentration, the greater the germicidal or bacteriostatic action in ointments. The *p*-hydroxybenzoates, being the most popular preservatives, have been very widely investigated (5, 6, 7) with the pH factor included in some of the works. Bandelin (8) tested 12 antifungal agents at various pH levels and found that organic acids lost activity as pH increases, while the *p*-hydroxybenzoates and esters of vanillin seemed to be affected little, if any, by pH variation. Aalto, *et al.* (9), also found the *p*-hydroxybenzoates to be substantially equally effective between pH 4 and 8, inclusive. The majority of reports concerning pH and antimicrobial activity shows that there is a general relationship between the two and this relationship is, the lower the pH the greater is the antimicrobial activity of the preservatives.

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EXPERIMENTAL

Trypticase soy broth was used as the media in this investigation for two reasons. It is a fairly good nutrient for many organisms, and the liquid state was desired over a solid material because liquids are inherently more difficult to preserve than solids or semisolids. Portions of this broth were prepared in McIlvane buffer at pH 2, 3, 4, 5, and 6. Five-milliliter portions of broth buffered to each pH level were placed in 13 × 100 mm. test tubes, and the tubes were stoppered with cotton plugs and sterilized. Autoclaving did not alter the pH of the buffered broth more than ±0.1 pH unit. These tubes of broth were then ready for addition of the preservative and subsequent inoculum.

The preservative materials under question were dissolved in alcohol or water, depending upon their respective solubilities. Their concentrations were adjusted so that 0.1 ml. of the solution contained the desired amount of preservative. Each material was tested at three concentrations selected to span its theoretical activity range. Then, 0.1 ml. of each of the three concentrated solutions of preservative was added to one buffered broth tube at each pH level. The control consisted of a buffered

broth tube with 0.1 ml. of alcohol or water added corresponding to the solvent used for the preservative. The controls showed that alcohol in the concentration of 2% (0.1 ml. in 5 ml. of broth) was not sufficient to cause any inhibition of growth. In some cases, the preservative materials were partially precipitated from solution at the higher concentrations upon addition of the concentrated preservative solution to the 5-ml. buffered broth tube. None of the preservative materials caused a significant change in pH of the buffered broth. After addition of the preservative solution and agitation of the buffered broth tubes to insure uniformity, the tubes were inoculated.

A soil sample, as a source of microbial inoculation, was chosen in preference to selected organisms because it was felt that the usual contamination of medicinals and foods is from this media. It was also felt that a larger variety of organisms would be encountered by this method. A preservative, to be truly useful, must be effective against all microorganisms and the results obtained from use of a soil inoculum represented activity against the greatest possible variety of contaminants. The soil sample was checked for organism content and there were found Gram-positive and Gram-negative spore-forming rods, cocci, yeast, and molds. In order to insure a large number of organisms the soil was fortified by adding 50 ml. of broth, buffered to the separate pH values, to each of five 100-Gm. portions and exposing these mixtures to the weather for one week. In future experiments the fortified soil samples were used to inoculate their respective buffered broth tubes. The inoculating suspension was prepared by adding approximately 250 mg. of each fortified soil sample to about 5 ml. of purified water. After sufficient agitation a standard loop of the suspension was transferred to each of the respective preserved buffered tubes and the control.

The inoculated preserved buffered broth tubes, hereafter designated IPBB, were examined periodically by checking for any of three conditions: visible growth, stasis, or germicidal effect. Visible growth was noted by clouding of the broth and subsequent precipitation. The stasis and germicidal effects were checked by transferring a standard loop of the IPBB not having visible growth to a check tube containing 4 ml. of trypticase soy broth at pH 7.3. These check tubes were watched for a minimum period of seventy-two hours before classifying the IPBB tubes. If the check tube showed visible growth the IPBB tube was classed as static; if no growth was observed in the check tube the IPBB tube was classed as germicidal. Originally the IPBB tubes were checked at twenty-four and seventy-two hours, one and two weeks, and one and three months. Later it was deemed sufficient to check them at one and two weeks and one and three months. In the tables, visible growth is denoted by a plus (+), stasis by a circled plus (⊕), and germicidal effect by dots (..).

Evaporation of the aqueous solvent from the tubes on long storage presented a problem as most of the water would dissipate in two to three months, giving a highly concentrated preservative solution and subsequent false impressions. This was overcome by periodically adding sterile distilled water to the tubes to keep the 5-ml. volume relatively constant.

In order to compare the broth media with something more nearly resembling a pharmaceutical product, the same test procedure was followed with the exception that a 50% sucrose solution and a 1% alginic acid-50% sucrose solution were used in two tests in place of the trypticase soy broth. The butyl-ethyl *para*-hydroxybenzoate mixture was chosen as the preservative materials for these tests.

DISCUSSION

Table I shows the effectiveness of methylparaben at each of the time intervals listed. In the early stages, as is usually the case, the preservative causes stasis but, on longer standing, this condition was converted to germicidal.

TABLE I.—METHYLPARABEN

pH	Blank	0.1%	0.2%	0.3%
		1 Week		
6	+	+	⊕	⊕
5	+	⊕	⊕	⊕
4	+	+	⊕	⊕
3	+	⊕	⊕	⊕
2	..	..	..	..
		2 Weeks		
6	+	+	⊕	⊕
5	+	..	⊕	⊕
4	+	+	..	⊕
3	+	⊕	⊕	⊕
2	..	..	..	..
		1 Month		
6	+	..	⊕	..
5	+	⊕	⊕	..
4	+	+	..	⊕
3	+	⊕	..	⊕
2	..	..	..	..
		3 Months		
6	+	+	..	..
5	+	..	..	..
4	+	+	..	..
3	+	..	..	⊕
2	..	..	..	..

Table II gives a comparison of the parabens. These results resemble, in general, the previously reported preservative activities of the parasepts based on pH, with the exception that the propyl and butyl esters were ineffective at pH 6 at almost maximum solubility.

Table III gives results with Chemo preservatives. *p*-Chlorophenylpropanediol showed rather uniform preservative activity at a concentration of 0.5% over the entire pH range used and also seemed to have the possibility of being effective at higher pH's. Salicylanilide seemed to be most effective at pH 5 or below. Cetyltrimethylammonium bromide showed very uniform results over the entire concentration and pH ranges and seemed to warrant extensive study.

Table IV shows results with sodium benzoate, mercuric chloride, sorbic acid, and dehydroacetic acid-sodium. Sodium benzoate and mercuric chloride preserved the broth as would be expected of them, sodium benzoate being effective at pH 5 and below, and mercuric chloride requiring a 1:25,000 concentration above pH 4. Sorbic acid appeared to

TABLE II.—COMPARISON OF PARABENS[a]

pH	Blank	0.1%	0.2%	0.3%
		Methylparaben		
6	+	+	..	..
5	+	..	..	..
4	+	+	..	..
3	+	..	..	⊕
		Ethylparaben		
pH	Blank	0.05%	0.1%	0.2%
6	+	+	⊕	..
5	+	+	..	..
4	+	+	..	..
3	+	..	..	..
		Propylparaben		
pH	Blank	0.025%	0.05%	0.075%
6	+	+	+	+
5	+	+	..	⊕
4	+	⊕	⊕	..
3	+	..	..	..
		Butylparaben		
pH	Blank	0.01%	0.02%	0.03%
6	+	+	+	+
5	+	+	+	⊕
4	+	..	..	..
3	+	..	..	..

[a] Preservatives courtesy of Heyden Newport Chemical Corp. Readings at three months.

TABLE III.—CHEMO PRESERVATIVES[a]

pH	Blank	0.05%	0.1%	0.5%
		p-Chlorophenyl Propanediol		
6	+	+	..	..
5	+	+	+	..
4	+	+	+	..
3	+	+	..	⊕
		Cetyltrimethylammonium Bromide		
pH	Blank	0.025%	0.05%	1%
6	+	..	..	..
5	..	..	..	..
4	+	..	..	..
3	+	..	..	..
		Salicylanilide[b]		
pH	Blank	0.02%	0.04%	.1%
6	+	+	+	⊕
5	+	..	..	⊕
4	+	..	..	..
3	..	..	..	..

[a] Preservatives courtesy of Chemo Puro. Readings at three months. [b] Exceeded solubility at highest per cent.

exert best preservative action at pH 5 and below, except at the highest concentration. Dehydroacetic acid-sodium preserved very well above a concentration of 0.2% and below pH 5.

Table V shows results with vanillic acid and esters. Vanillic acid was ineffective as a preservative but the esters showed good results in some areas. Just as with the *p*-hydroxybenzoate esters the lowest molecular weight material was least affected by pH change and activity decreased with increase in molecular weight. The propyl and isobutyl esters were not active at pH 6.

Table VI shows results with parabens combinations. The combinations of parabens in broth were inconsistent in their results. The methyl-propyl combination didn't preserve as well as the methyl alone and was comparable to the propyl

TABLE IV.—SODIUM BENZOATE, MERCURIC CHLORIDE, SORBIC ACID, AND DEHYDROACETIC ACID-SODIUM

pH	Blank	0.1%	0.2%	0.3%
		Sodium Benzoate		
6	+	+	+	+
5	..	..	..	..
4	+	..	..	..
3	+	..	..	..
		Sorbic Acid[a]		
pH	Blank	0.05%	0.1%	0.2%
6	+	+	+	..
5	+	..	..	..
4	+	⊕	..	..
3	+	..	..	..
		Mercuric Chloride		
pH	Blank	1:50,000	1:25,000	1:5,000
6	+	+	..	..
5	+	+	+	..
4	+	..	..	..
3	+	..	..	..
		Dehydroacetic Acid-Sodium[b,c]		
pH	Blank	0.1%	0.2%	0.5%
6	+	+	..	..
5	+	..	..	..
4	+	..	..	⊕
3	+	..	..	..

[a] Courtesy of Union Carbide Chemicals Co. [b] Courtesy of Dow Chemical Co. [c] Exceeded solubility at highest per cent. Readings at three months.

TABLE V.—VANILLIC ACID AND ESTERS[a]

pH	Blank	0.1%	0.4%	0.8%
		Vanillic Acid, Ethyl Ester[b]		
6	+	+	⊕	..
5	+	..	..	..
4	+	..	..	..
3	+	..	..	..
		Vanillic Acid		
pH	Blank	0.04%	0.1%	0.2%
6	Discontinued at one month since all tubes contained visible growth.			
5				
4				
3				
		Vanillic Acid, Isobutyl Ester[b]		
pH	Blank	0.1%	0.4%	0.8%
6	+	+	+	+
5	+	⊕	+	+
4	..	..	..	..
3	+	..	..	..
		Vanillic Acid, Propyl Ester[b]		
pH	Blank	0.1%	0.4%	0.8%
6	+	+	+	+
5	+	⊕	..	..
4	..	..	⊕	..
3	+	..	..	..

[a] Preservatives courtesy E. R. Squibb and Sons. Readings at three months. [b] Exceeded solubility at highest per cent.

alone, not being effective at pH 6. The ethyl-butyl combination was an improvement over either material alone, showing synergistic or potentizing effect. The ethyl-butyl combination in 50% sucrose solution seemed to preserve better than in broth.

Table VII shows results obtained with hexachlorophene and hexylresorcinol. Hexachlorophene and hexylresorcinol were completely effective at all concentrations tested and over the entire pH range used.

TABLE VI.—PARABENS COMBINATIONS[a]

pH	Blank	0.1%M 0.025%P	0.2%M 0.05%P	0.3%M 0.075%P
Methyl and Propyl Parabens				
6	+	+	+	+
5	+	⊕	⊕	⊕
4	+	..	..	..
3	+	..	..	..
		0.05%E 0.01%B	0.1%E 0.02%B	0.2%E 0.03%B
Ethyl and Butyl Parabens				
6	+	+	⊕	⊕
5	+	⊕	..	..
4	+	..	..	..
3	+	..	..	..
Ethyl and Butyl Parabens (in 50% Sucrose)				
6	+	⊕	⊕	⊕
5	+	⊕	⊕	⊕
4	+	⊕	⊕	⊕
3	+	..	⊕	⊕
Ethyl and Butyl Parabens (in 50% Sucrose–1% Alginic Acid)				
6	+	..	..	⊕
5	+	⊕	⊕	..
4	+	⊕	⊕	..
3	+	..	⊕	..

[a] Preservatives courtesy of Heyden Newport Chemical Corp. Readings at three months.

TABLE VII.—HEXACHLOROPHENE AND HEXYLRESORCINOL[a]

pH	Blank	0.025%	0.05%	0.1%
Hexachlorophene[b]				
6	+	..	..	..
5	..	..	..	..
4	⊕	..	..	..
3	..	..	..	..
		0.02%	0.05%	0.1%
Hexylresorcinol[b]				
6	+	..	..	..
5	+	..	..	..
4	+	..	..	..
3	+	..	..	..

[a] Readings at three months. [b] Exceeded solubility.

SUMMARY

Several commonly used and new materials have been checked for preservative effectiveness at pH's between 2 and 6, inclusive. At pH 2 organisms died without preservative materials being present. Therefore, it was concluded that this pH alone is sufficient to preserve.

A fortified soil sample was used as inoculum in an effort to check preservative effectiveness against large numbers of various organisms. Trypticase soy broth was used as media for this investigation.

Results show some deviation from previously reported activity of some preservatives as pH varies while others correspond rather closely. Some new materials seem to warrant further investigation while others appear worthless. A comparison between the trypticase soy broth and a syrup as media was made with results indicating that the broth is a better test media.

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Pharmacologic Study of Alkyl 2-Bromoallyl Barbituric Acids

By HARALD G. O. HOLCK, MARVIN H. MALONE†, and HIDEKO KATAYAMA KAJI‡

The *n*-propyl-(PBB), ethyl-(EBB), and methyl-(MBB) homologs of propallylonal(IPBB) were prepared. Using albino rats, the intraperitoneal ED_{50}s and LD_{50}s were determined, as well as PD_{50}s against pentylenetetrazol. Effectiveness in developing tolerance and cross-tolerance against propallylonal by feeding low concentrations in the diet for two weeks was in decreasing order: EBB, probarbital, IPBB, PBB, MBB, and 5-(2-bromoallyl)-5-(1-methylbutyl)barbituric acid. Inter- and intralaboratory variations occurred as to incidence of delayed death (EBB, IPBB) and in sex variation (IPBB). Rat electrocardiograms taken after administration of the bromoallyl barbiturates revealed no significant changes. In mice, PBB was most effective and IPBB least effective in protecting against a surely fatal dose of strychnine when given at various intervals of time after the barbiturate. None of the bromoallyl barbiturates gave complete protection when given one hour before a dosage range of 2.5–20.2 mg./Kg. of strychnine sulfate subcutaneously. Phenobarbital sodium was superior to the bromoallyl compounds against strychnine lethality.

PROPALLYLONAL or 5-(isopropyl)-5-(2-bromoallyl) barbituric acid is an unusual hypnotic in that injection of hypnotic doses usually causes a high incidence of delayed deaths in rats one to five days later. Such deaths may even follow subhypnotic doses (1, 2). This death is commonly accompanied by pronounced edema of the lungs with labored respiration, by hepatization, and by pneumonia. Fatty infiltration of the liver, kidneys, heart, and lungs, ataxia, anorexia, and emaciation may also occur. Delayed deaths following propallylonal did not occur in the earliest experiments done with the rat according to Vogt (3); however, Boedecker and Ludwig (4) reported occasional delayed deaths in the rabbit after large oral doses of a homolog, butallylonal. Fitch and Tatum (1) described delayed effects in the rat leading to death in a few days, and Holck and co-workers carried out a series of investigations dealing with the consistent occurrence of delayed death in a variety of albino and hybrid rat stocks in Beirut, Chicago, and Lincoln, and pointed out the variable sex variation shown by the rat to this barbiturate. The phases studied have dealt with: (*a*) the age factor (2); (*b*) the characteristic pathological changes induced (2); (*c*) the effects of modifying the isopropyl group, showing that lengthening it to the 2-butyl or 2-amyl chain diminished the tendency towards delayed death, an effect also noted with the N-methyl homolog [5-(2-bromoallyl)-5-isopropyl-1-methylbarbituric acid] (2); and simultaneously, the hypnotic action with all three was markedly shortened and the male resistance compared to that of the female enhanced (5); (*d*) the lack of influence exerted by monogamy, polygamy, celibacy, various stages of pregnancy (6), and repeated administration of testosterone propionate (7); (*e*) the effect of partial hepatectomy or liver injury induced by carbon tetrachloride to prolong the propallylonal depression time, while feeding several desiccated liver preparations did not influence incidence of delayed death (8); (*f*) hypothyroidism produced by thyroidectomy or by feeding propylthiouracil also lengthened the depression time and thyroidectomy of male rats appreciably increased the incidence of acute death; also, delayed deaths occurred sooner after intraperitoneal injection of a propylthiouracil suspension one hour prior to propallylonal injection, yet this did not influence the number of either the acute or delayed deaths (9); (*g*) a thorough analysis was made of the tolerance and cross-tolerance rapidly induced by injecting several small doses or feeding low concentrations of propallylonal and some related barbiturates, notably isopropyl ethyl barbituric acid, probarbital (10).

The present study was undertaken to elucidate

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† Present address: School of Pharmacy, University of Connecticut, Storrs.

‡ Present address: Vanderbilt Medical School, Nashville, Tenn.

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TABLE I.—PHYSICAL AND CHEMICAL PROPERTIES OF PROPALLYLONAL AND ITS *n*-PROPYL, ETHYL, AND METHYL HOMOLOGS

Code Name	Chemical Name	Molecular Weight	Observed M. P.° Range	% Nitrogen Found[a]	% Nitrogen, Calcd.
IPBB	5 - (Isopropyl) - 5 - (2 - bromoallyl) barbituric acid; propallylonal	289.14	177–178	9.34	9.70
PBB	5 - (*n*-Propyl) - 5 - (2 - bromoallyl) barbituric acid	289.14	186–188	9.37	9.70
EBB	5 - (Ethyl) - 5 - (2 - bromoallyl) barbituric acid	275.11	174–176	9.74	10.18
MBB	5 - (Methyl) - 5 - (2 - bromoallyl) barbituric acid	261.08	180–182	10.16	10.73

[a] Nitrogen analyses were conducted by the Clark Microanalytical Laboratory, 104½ West Main Street, Urbana, Ill.

further the influence of slight variations in chemical structure upon acute and delayed effects, using the methyl, ethyl, and *n*-propyl homologs of propallylonal and this drug itself.[1] See Table I. Several attempts to introduce either iodine or fluorine into the allyl radical were unsuccessful.

EXPERIMENTAL

Nebraska stock rats were raised in the animal quarters of this laboratory and bred originally from Wistar stock. After sex separation at the age of four to six weeks they were housed in colony cages; temperature of the quarters was maintained at approximately 25°. Unless specified, their diet consisted of Purina powdered laboratory chow and water *ad libitum*. Canada stock rats, likewise bred from the Wistar strain, were donated by Dr. M. K. Allmark of the Food and Drug Division, Canadian Department of National Health and Welfare. Carworth stock rats, originally of the Wistar stock, were purchased from the Carworth Farms, New City, N. Y. Prior to tests, rats from these colonies were maintained for at least eighteen days under conditions identical to those described for Nebraska stock rats. All intraperitoneal injections were made in the upper left quadrant of the abdomen, while all subcutaneous injections were made in the lower abdominal region. During the acute tests each animal was kept in an individual cage and the temperature of the laboratory controlled at 25 ± 1°. An animal was considered to be asleep when the righting reflex had been lost and the animal would lie quietly on either side (RRL). Recovery (R) was considered complete when the animal promptly retracted both hind legs from an extended position. Duration of lost reflex was the time from RRL to R, and acute death time was the time from injection until death. After testing for acute effects, rats were observed for at least five days to record the incidence of delayed death; they were not used for further experimentation unless specified. Shortly before administration, each bromoallyl barbiturate was converted to its sodium salt by the addition of an appropriate amount of 0.2 *N* sodium hydroxide and adjusting to the desired volume with freshly boiled, triple distilled water.

[1] The homologs were synthesized by David B. Capps and Joe R. Willard of the Avery Laboratories, University of Nebraska.

RESULTS AND DISCUSSION

Effective, Protective, and Lethal Dose Determinations.—Propallylonal and its *n*-propyl, ethyl, and methyl homologs will hereafter be designated by their code names; IPBB, PBB, EBB, and MBB, respectively. After fasting overnight with water allowed *ad libitum*, and after randomization, 5 to 6 rats per sex per dosage were injected with an appropriate logarithmic series of doses: the dosage increment used was 10.4% (log 0.0430) or 12.3% (log 0.0502). Calculations following the method of Reed and Muench (11) and that of Pizzi (12) were made to determine each compound's ED_{50} (dose producing RRL in 50%) and LD_{50} (dose acutely lethal to 50%). The PD_{50} was also determined for each test compound; this was the dose that would protect 50% of the rats from epileptiform convulsions caused by 70 mg./Kg. of pentylenetetrazol injected subcutaneously as a 5% solution one hour after intraperitoneal injection of the respective barbiturates. Results are tabulated in Table II.

The hypnotic potencies of IPBB, PBB, and EBB were, respectively, 3.8, 2.5, and 2.4 times that of MBB. As expected, MBB had the weakest depressant action as shown by the LD_{50}, ED_{50}, and PD_{50} values; it also had the longest induction time and the longest duration of action. Although EBB appeared to be more potent as judged by a lower LD_{50}, the hypnotic strength was practically equal to that of PBB, as judged by their ED_{50}s. The fork-chained IPBB, as expected, had the most rapid action and produced the lowest LD_{50} and ED_{50} values. However, the efficiencies in antidoting pentylenetetrazol did not relate directly to either ED_{50}s or LD_{50}s in case of the three most effective substances, in that the PD_{50} dose was lowest for EBB, followed by IPBB and PBB. As to their relative safety, however, PBB was superior when judged by its LD_{50}/ED_{50} ratio, but EBB ranked highest when the LD_{50}/PD_{50} ratio was considered. Moreover, although IPBB ranked second in regard to the first of these criteria, it fell far below the others in safety when judged by the second, because often a dose greater than that abolishing the righting reflex was required to prevent convulsions. No sex variation was seen either in regard to the quantal incidence of narcosis or to the incidence and onset of acute and delayed death; however, females given nearly lethal doses of IPBB recovered considerably more slowly than the males at the same dosage level. The analeptic effects of pentylenetetrazol did not prevent

TABLE II.—MEDIAN INTRAPERITONEAL ACUTE LETHAL, EFFECTIVE, AND PROTECTIVE (MG./KG.) DOSES OF ISOPROPYL, PROPYL, ETHYL, AND METHYL 2-BROMOALLYL BARBITURIC ACIDS AND THEIR RATIOS IN RATS[a]

	IPBB	PBB	EBB	MBB
LD_{50}	96.8 ± 3.7[b]	177.0 ± 7.0	140.1 ± 5.7	325.7 ± 13.9
	(60)12[c]	(60)12	(40)10	(60)12
ED_{50}	30.5 ± 1.2	46.7 ± 1.6	47.6 ± 1.4	116.8 ± 3.5
	(50)10	(72)12	(40)10	(48)12
PD_{50}	38.7 ± 2.0	44.0 ± 1.7	32.7 ± 1.4	82.3 ± 2.7
	(100)10	(84)12	(70)10	(60)12
LD_{50}/ED_{50}	3.2	3.8	2.9	2.8
LD_{50}/PD_{50}	2.5	4.0	4.3	4.0
ED_{50}/PD_{50}	0.8	1.1	1.5	1.4

[a] Four- to six-months old Nebraska stock albino rats were used. [b] Standard error. [c] Numbers in parentheses represent rats included in groups not having either 100% negative or 100% positive responses. Numbers following these indicate the number of rats per dosage group.

TABLE III.—EFFECTS OF FEEDING EQUIMOLECULAR CONCENTRATIONS OF SELECTED BARBITURATES FOR TWO WEEKS UPON THE RESISTANCE OF CANADA RATS TO 90 MG./KG. (A) AND OF CARWORTH RATS TO 135 MG./KG. (B) OF ISOPROPYL-2-BROMOALLYL BARBITURIC ACID ADMINISTERED SUBCUTANEOUSLY[a]

Group	Drug per Kg. of Powdered Food	Average Weight, Gm. Initial	Final	No. of Rats	Quantal Loss of Righting Reflex	Mean Recovery Time, min.	Acute Deaths	Delayed Deaths
A	Food only	157	187	11	11	90(9)[b]	2	0
B		149	198	16	16	279(6)	10	6
A	200 mg. of IPBB	160	187	12	0	...	0	0
B		150	201	15	8	54(8)	0	0
A	200 mg. of PBB	155	184	12	1	49(1)	0	0
B		153	200	14	3	115(3)	0	5
A	190.3 mg. of EBB	156	184	12	0	...	0	0
B		151	209	13	0	...	0	0
A	180.6 mg. of MBB	161	187	12	1	54(1)	0	0
B		151	204	15	12	104(11)	1	3
A	152.3 mg. of probarbital sodium	164	190	11	0	...	0	0
B		149	203	15	0	...	0	2
B	234.9 mg. of 5-(2-bromoallyl) - 5 - (1 - methylbutyl) barbituric acid sodium	148	205	16	16	196(11)	5	6

[a] The IPBB or propallylonal was administered as a 1.5% solution. The Canada male albino stock rats were fourteen to fifteen weeks old and the Carworth male albino rats were eleven weeks old at the time of the final propallylonal injection. [b] Numbers in parentheses indicate the number of rats upon which the mean is based.

delayed death with IPBB in the dosage range where no acute deaths occurred. Thus, in the range of 23–37 mg./Kg. dosage of IPBB (60 rats), five such deaths were recorded, one where the righting reflex was not lost; and with the 41–61 mg./Kg. dosage range (50 rats), 16 such deaths occurred. Of the 21 delayed deaths, 10 occurred in male and 11 in female rats. All were recorded on the fourth or fifth day after dosage. Yet with PBB (84 rats), EBB (90 rats), and MBB (96 rats) in the nonfatal, but pentylenetetrazol-protective dosage range, no delayed deaths were noted, even where the righting reflex had been lost by all the rats in a group.

Tolerance and Cross-Tolerance.—Following the techniques of Holck, *et al.* (10), powdered food containing equimolecular concentrations of the test barbiturates was fed to Canada stock rats for fourteen days prior to dosage with 90 mg./Kg. of IPBB. This subcutaneous dosage in Nebraska stock rats regularly has induced deep depression, with or without acute death, but with high incidence of delayed death (10). All rats gained weight to about the same extent during the feeding period. They were not fasted prior to IPBB injection. Results are tabulated in Table III, group A.

None of the rats receiving IPBB, EBB, or probarbital in the food lost the righting reflex and only one animal per group of those fed PBB and MBB did this in contrast to the 100% loss in the control group. However, the complete absence of delayed death in the control group was unexpected and appeared to indicate that Canada stock rats were very resistant to IPBB-delyead death. Assuming that any tolerance or cross-tolerance to IPBB would be absent after twenty days rest (10), rats surviving this experiment were randomized with respect to previous feeding treatment, arranged into groups, and again maintained on the test diets for fourteen days. At the end of this period they were injected subcutaneously with a higher dose (120 mg./Kg.) of IPBB. The rats gained 7–9% in body weight during the feeding period. Those receiving IPBB, PBB, and MBB in their food were quite depressed with this higher dosage of IPBB, but in only the first group did a number of rats lose the righting reflex. Rats consuming PBB in their food were only slightly depressed and those getting probarbital were not noticeably affected. There was no adequate explanation for the lack of increase in mean recovery time for this control group (eighty-seven minutes) as compared with that of the controls for the previous test (ninety minutes) that had re-

ceived only 90 mg./Kg. or 75% of the new dose. Again, no acute deaths occurred in any group; however, one male in each of the groups fed IPBB and PBB succumbed to a "typical" delayed death.

To observe if any effect would remain from the two previous doses of 90 and 120 mg./Kg. of IPBB, each given after two weeks of drug-feeding, 90 mg./Kg. of IPBB were again injected subcutaneously, after allowing three months for full recuperation from the last 120 mg./Kg. dose. The average weight (259 Gm.) of these rats was identical with and the age nearly the same as that of a control group of previously untreated Canada stock male rats which were now dosed with 90 mg./Kg. of IPBB. The mortality rate was higher in the premedicated rats, in that seven di d as compared to four out of 13, and the average depression of righting reflex was two hundred and fifteen minutes for the remaining survivors as compared to one hundred and thirty-one minutes for the fresh stock survivors. Also, three died delayed deaths on the fifth, eighth, and tenth day after dosage, respectively. In spite of the long rest allowed, a loss in resistance to IPBB had been produced in these originally resistant Canada stock rats, apparently due to incomplete tissue recovery from the previous large doses of IPBB.

Because of a shortage of Nebraska stock rats, Carworth stock rats were obtained, maintained on the test diets for fourteen days, and then injected with 135 mg./Kg. of IPBB, a dose previously found to be uniformly fatal to Nebraska stock rats (10). The average weight gains ranged from 30–39%, those in the control group gaining 32%. Results are tabulated in Table III, group B. Results indicated that feeding EBB was even more effective than probarbital and that both of these were superior to IPBB in establishing tolerance or cross-tolerance to IPBB. Although PBB and MBB prevented loss of righting reflex and shortened the depression time of those rats that did lose it, neither completely prevented mortalities after the IPBB dose that killed all of the controls. All three homologs of IPBB were superior to 5-(2-bromoallyl)-5-(1-methylbutyl) barbituric acid, the 2-amyl homolog, in establishing cross-tolerance to IPBB. Post-mortem examination after delayed death revealed typical hepatized lungs (2). Thus, Carworth stock rats were about as susceptible to IPBB as Nebraska stock rats and the qualitative response pattern was similar.

Variations in Response to IPBB and EBB.—In an attempt to elucidate the matter of colony variation in rat response to IPBB, Dr. Allmark carried out supplementary tests in Ottawa and has allowed presentation here of his experimental data upon Canada Wistar and Sprague Dawley stock rats, the latter obtained locally in Canada. His results are presented in Table IV for comparison with our data.

Male Canada Wistar stock rats tested in Ottawa had a longer period of narcosis with essentially the same quantal incidence of acute death as did male Canada Wistar stock rats when injected with a similar dosage in Lincoln. However, delayed deaths occurred at all three dosage levels in the Ottawa tests, but not at Lincoln, where only the highest dosage was administered, even though some of these Canada stock rats had been placed on our Nebraska colony diet for several months prior to IPBB medication. Young female Canada stock rats were much more susceptible than males to IPBB as judged both by incidence of acute and delayed death; also, on an average, delayed deaths in the females came one day earlier than in the case of the males (1.8, 2.9 days, respectively). No such variation was seen in the more mature Nebraska stock rats. Both sexes of

Table IV.—Interlaboratory Variation in Lethality after IPBB Testing in Several Albino Rat Stocks and Intralaboratory Variation after EBB in One Rat Stock

Rat Stock	Age, wk.	Dosage, mg./Kg.	Route of Injection	Males			Females		
				No. Rats	No. of Deaths Acute	No. of Deaths Delayed	No. Rats	No. of Deaths Acute	No. of Deaths Delayed
Experiments with IPBB at Ottawa									
Canada Wistar	12–13	60[a]	s.c.	25	0	2	25	0	8
		75[a]	s.c.	25	2	2	25	9	10
		90[a]	s.c.	25	9	4	25	20	3
			Total	75	11	8	75	29	21
Sprague Dawley	7–8	60	s.c.	10	0	6	10	0	9
		75	s.c.	10	0	9	10	0	10
		90	s.c.	10	0	9	10	3	7
			Total	30	0	24	30	3	26
Experiments with IPBB at Lincoln									
Canada Wistar	14–15[b]	90[a]	s.c.	23	5	0	..	..	..
	28–31[c]	90	s.c.	13	4	0	..	..	..
Nebraska Wistar	13–15	90	s.c.	12	0	7	..	..	..
	16–25	71	i.p.	6	0	4	6	0	3
	16–25	79	i.p.	6	0	5	6	1	3
	16–25	89	i.p.	6	2	4	6	3	3
	16–25	100	i.p.	6	4	2	6	3	2
			Total	24	6	15	24	7	11
Carworth Wistar	12–13	135	s.c.	16	10	6	..	..	..
Experiments with EBB at Lincoln									
Nebraska Wistar	16–25	123–191	i.p.	36	19	0	36	16	0
	16–20	115–174	i.p.	20	12	0	20	9	0
	16–25	112–178	i.p.	30	17	8	20	8	4

[a] Combination of data of two separate tests at different times. [b] Rats were fed on Nebraska colony diet four to six weeks after arrival and prior to testing. [c] Rats were fed on Nebraska colony diet for four months after arrival and prior to testing.

the Sprague Dawley rats recovered faster and acute deaths occurred only with females given the highest dose of IPBB. However, the incidence of delayed death was very high and present with equal frequency in both sexes, although the females died, on an average, one day earlier than the males (1.5, 2.4 days, respectively).

There is no apparent explanation for the enigma that Canada Wistar stock male rats showed no incidence of delayed death following IPBB dosage at Lincoln (even though they had been housed at Lincoln, and fed the Nebraska colony diet for several months), but did show this ability at Ottawa (where the rat diet resembled the Nebraska colony diet). The laboratory temperature at Ottawa differed too slightly from that of our laboratory (23.5 *vs.* 25°) to be of significance.

Also unexplainable is the fact that in our first two experiments with EBB no delayed deaths followed its administration; whereas, in the third test (Table IV), delayed deaths followed the usual pattern of frequency seen with IPBB. Change from one commercial diet (Crete Mills chow) to another (Purina laboratory chow) between the second and third EBB experiments cannot be an important factor because no delayed deaths occurred with IPBB after prolonged feeding of the Purina chow to Canada stock rats at Lincoln; also, the acute LD_{50}s of EBB calculated from the second and third tests were identical (140, 141 mg./Kg., respectively) indicating a generally similar resistance to EBB.

Electrocardiographic Comparison.—Pathology reports have indicated that the degree of fatty changes found in the myocardia of rats receiving IPBB would be considered significant in human necropsy material (2). A selective toxicity to the heart could be a contributing factor in producing the pulmonary edema characteristic of the IPBB delayed death. The converse could also be true.

One female and two male Sprague Dawley rats were injected intraperitoneally with each of the bromoallyl barbiturates and electrocardiograms were taken three, thirty-four, forty-eight, and seventy-two hours later. The mg./Kg. doses injected were 70, 120, 105, and 210 for IPBB, PBB, EBB, and MBB, respectively. These doses were surely hypnotic doses and approximately 70% of the respective LD_{50}s; with IPBB and EBB, delayed death might be expected. Five days after drug administration, all animals were autopsied and the lungs examined for gross evidence of pathological changes. Electrocardiograms using leads I, II, and III were obtained at the four specified times. All animals receiving IPBB showed mottled lungs on autopsy; only one rat receiving EBB and one receiving MBB had spotted lungs. The remaining rats were grossly normal. No delayed deaths occurred prior to autopsy. Slight disturbances in the electrocardiograms lasting one to two days were observed but they returned to normal within three days. A depression of the T-wave noted in some of the rats persisted after recovery time from acute depression in contrast to its disappearance just after anesthesia in larger animals given thiopental or hexobarbital (13). In spite of evidence of pathologic changes in the lungs, the electrocardiograms did not reveal any significant alterations in the conduction system or myocardium. No arrhythmias, extra systoles, exaggerated, or depressed waves were noted that might have emanated from pulmonary complications. The typical delayed death after IPBB is apparently not directly due to cardiotoxicity.

Determination of Antidotal Potency Against Strychnine Lethality in Mice.—Male Webster Swiss mice were obtained from John C. Landis Co., Hagerstown, Md. and were maintained on Purina laboratory chow checkers and water *ad libitum* in our animal quarters for at least one week prior to test. Just before injection, the mice were weighed to the nearest one-half gram and randomized within a weight range of 18–27 Gm. Strychnine sulfate injections were made subcutaneously in the lower back area and all barbiturates were given intraperitoneally. While on test, mice were kept in individual large beakers.

Using 70 mice, the surely fatal subcutaneous dose (LD_{100}) of strychnine sulfate was established to be 2.52 mg./Kg.; $^{10}/_{20}$ died receiving 2.00 and 2.24 mg./Kg., respectively, whereas 2.52 mg./Kg. killed $^{10}/_{10}$. In repeated control tests, this last dosage has always proved to be 100% fatal.

Experiments for each of the test barbiturates and phenobarbital were carried out to determine the maximal dose of these which would allow survival of all mice; this maximum nonlethal dose was considered suitable as a dose to antidote strychnine. Dosage increments were 12.3% (log 0.0502). The maximal nonlethal doses in mg./Kg., based upon the results of these tests, were estimated to be: phenobarbital sodium, 178.2 (4 groups, 31 mice); IPBB, 100.0 (4 groups, 40 mice); PBB, 200.0 (6 groups, 63 mice); EBB, 158.7 (5 groups, 50 mice); and MBB, 317.5 (4 groups, 40 mice). Observations for acute death were limited to forty-eight hours after injection. The phenobarbital sodium dosage is two-thirds that of two closely-agreeing, independent LD_{50} determinations in two laboratories using Swiss albino mice (14). This same approximate ratio holds for EBB and PBB, using our own data. In cases where mice were still sleeping on the day following barbiturate administration, one to two subcutaneous injections of sodium chloride solution, U. S. P. XV, in quantities corresponding to 1% of body weight were made to offset the effects of dehydration.

The first phase of this antagonism study was the determination of the degree of protective action of the barbiturates against one LD_{100} of strychnine sulfate, when the barbiturates were given at six intervals of time, ranging from eight hours to immediately

TABLE V.—INFLUENCE OF TIME INTERVAL BETWEEN BARBITURATE AND STRYCHNINE INJECTION UPON MORTALITY AFTER INJECTION OF THE SURELY FATAL DOSE OF STRYCHNINE TO MALE ALBINO MICE[a]

Hours After Barbiturate, i.p.	Mortality Per Group of Five Mice				
	Phenobarbital Sodium 178.2[b]	IPBB 100.0[b]	PBB 200.0[b]	EBB 158.7[b]	MBB 317.5[b]
At once	0	3	0	0	1
0.5	..	1	0	2	2
1	0	2	1	2	3
2	..	4	0	0	2
4	0	5	4[c]	3	3
8	0	5	5	5	5

[a] Dosage of strychnine sulfate was 2.52 mg./Kg., subcutaneously, 10 ml./Kg. [b] Mg./Kg. dosage of the barbiturates; equimolecular concentrations of the barbiturates were used, based upon a 4% solution of IPBB. [c] One mouse died in less than four hours; another was substituted.

TABLE VI.—MORTALITIES FROM STRYCHNINE SULFATE ONE HOUR AFTER INTRAPERITONEAL INJECTION OF PHENOBARBITAL SODIUM, IPBB, PBB, EBB, AND MBB TO MALE ALBINO MICE

Strychnine Sulfate mg./Kg.[a]	Phenobarbital Sodium 178.2[b]	Phenobarbital Sodium 158.7[b]	IPBB 100.0[b]	PBB 200.0[b]	EBB 158.7[b]	MBB 317.5[b]
2.52	0[c]	...	2	1	2	3[d]
3.56	...	...	4	2	0	2
5.04	...	...	5 + 2	2	4	5 + 4
7.12	...	...	5 + 4	3 + 2	4	...
10.18	0	...	...	2 + 2	5 + 4	...
14.24	0 + 0	...	...	2 + 5	...	...
20.16	2 + 1	1 + 2	...	4 + 5	...	...

[a] Dosage volume of strychnine sulfate was 10 ml./Kg. subcutaneously.
[b] All doses are in mg./Kg.; equimolecular concentrations of the barbiturates were used based upon a 4% solution of IPBB, except that a 7.22% solution of MBB was used.
[c] Each figure represents the number that died out of five mice; a second figure represents the mortality from an additional experiment upon five mice.
[d] One mouse died in less than an hour and another was substituted.

before the injection of strychnine. With strychnine administered one hour and also immediately after the barbiturates, additional tests of the protective action were carried out administering one-half of the estimated nonlethal barbiturate dosage. The results are summarized in Table V.

Varying degrees of protection were obtained even at the height of hypnotic action of the bromoallyl barbiturates and in all cases protection had ceased eight hours after the barbiturate administration and was clearly less and always incomplete at the four-hour interval. The order of efficiency was: PBB (most efficient), EBB, MBB, and IPBB. In contrast to these, phenobarbital sodium afforded full protection at all time intervals and even in half-dosage. When strychnine was administered immediately following the barbiturates in doses equal to one-half those shown in Table V or one hour after such injection, protection was complete only in the case of phenobarbital sodium; when strychnine was given immediately after MBB, no protection was afforded.

The second phase of this experiment dealt with a comparison of the relative effectiveness of the bromoallyl barbiturates against one to eight fatal doses of strychnine sulfate. For the first test, groups of five mice each were used; to make the comparisons more valid, additional groups of five were added where necessary until a total mortality of $^9/_{10}$ had been reached. Results are given in Table VI.

Even with the lower doses of strychnine sulfate the protection afforded by the bromoallyl barbiturates was usually incomplete. The antidotal potency against high dosage of strychnine sulfate was greatest for PBB, with EBB ranking second; IPBB and MBB were poor antidotes. Again phenobarbital sodium was markedly superior and gave complete protection against 4 and 5.6 surely fatal doses of the convulsant and a high degree of protection against 8 such doses, even when administered at 89% of the maximum nonlethal phenobarbital sodium dosage.

SUMMARY

1. The *n*-propyl homolog of propallylonal had the highest safety margin (LD_{50}/ED_{50}) in rats, was fairly effective in establishing cross-tolerance to propallylonal and ranked highest in protective power against strychnine sulfate in mice, though not protecting all mice from a normally fatal dose.

2. The ethyl homolog was safest judged by the LD_{50}/PD_{50} index, was most effective in inducing cross-tolerance to propallylonal, and ranked second as an antidote to strychnine sulfate. Delayed deaths followed its administration in only one of three tests.

3. The methyl homolog had the weakest hypnotic potency, was fourth in safety margin using the LD_{50}/ED_{50} criterion, but had the same safety index as the *n*-propyl derivative when the LD_{50}/PD_{50} ratio was considered. It was a poor strychnine antidote.

4. Propallylonal was the most potent, ranked poorest in safety as judged by the LD_{50}/PD_{50} index, but was second best considering only the LD_{50}/ED_{50} ratio. It was very efficient in establishing tolerance to itself and was very poor as a strychnine antidote. Delayed death after propallylonal was observed in four different rat colonies but the degree and nature of the response appeared to vary with both the rat colony and the locale of the experiment. The phenomenon of delayed death was not due to a direct cardiotoxicity.

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Interfacial Properties of Powdered Material; Caking in Liquid Dispersions II

Electrokinetic Phenomena

By BERNARD A. HAINES, Jr.† and ALFRED N. MARTIN

Electrokinetic characteristics of insoluble particles in dispersion were shown to be dependent upon the existing charge on the particle surface as related to the polarity of the dispersing solvent. A procedure is described for observing the electrophoretic mobility of charged particles from which the zeta potential can be calculated. The magnitude of the zeta potential was used as an index of caking. The electrical discharge of particles by flocculating techniques led to a reduction in the caking tendency of the dispersed particles.

IN A PREVIOUS study (1) it was found that sulfamerazine suspensions would not cake when flocculated with aluminum ions. Similarly, bismuth subnitrate suspensions did not cake when flocculated with phosphate ions. Flocculation is considered to be due to the electrical discharge of the particle surfaces by the oppositely charged ions. Therefore, if the decrease in caking tendency can be attributed to the electrical discharge of dispersed particles, the electrokinetic potential existing in the system should be indicative of the state of dispersibility of the system. This study was conducted to determine the relationship between electrokinetic potential and the caking tendency of particles in dispersion.

Particles dispersed in a solvent acquire either a positive or negative charge, depending upon the nature of the particles and the polarity of the solvent. The charge originates either by surface ionization or by adsorption of ions from solution. Portions of the molecule containing amine, carboxyl, or other ionizable groups determine the charge of the particle. The pH, ionic strength, and degree of hydration by the solvent also contribute to the charge of a particle.

A potential difference arises between a dispersed particle and its dispersion medium, due to the unequal tendency of the ions to distribute themselves between the phases. This potential was first described by Freundlich (2), who called it the zeta potential, ζ. The potential assumes the sign of the ions preferentially adsorbed on the surface of the particle. The sign may be positive, negative, or zero, depending on the ions that influence the system.

In suspensions composed of hydrophobic powders dispersed in water, the magnitude of repulsion between particles is great as shown by electrokinetic observations. The electrophoretic mobility of charged particles is usually high and the tendency to flocculate is small or completely absent. Accordingly, the electrophoretic mobility of flocculated particles is greatly reduced or, in some instances, does not exist. The zeta potential, which is the potential drop across the electrical double layer at the interface between the colloidal particles and the suspending medium, is a measure of this mobility and also of the degree of flocculation. This potential may be markedly affected by small amounts of selected electrolytes, reducing the charge on the particle sufficiently to allow the formation of floccules.

A measurement of the electrokinetic or zeta potential of the particles can be made by observing the movement of a charged particle under the influence of an applied electromotive force. This electrokinetic phenomenon, which yields information relative to the charge on the particles, is known as electrophoresis. The velocity with which a charged particle moves in an electrical field depends upon the intensity of the electrical field in which the system is operating and is expressed in terms of electrophoretic mobility, the velocity a particle travels in centimeters per second through a system across which a potential of one volt per centimeter is operating.

PROCEDURE

Microscope Electrophoresis.—A modified Northrop-Kunitz (3–5) type electrophoresis assembly, consisting of an electrophoresis cell, a microscope, an electrode system, provisions for filling and emptying the cell, and an electronic circuit for reading voltages applied to the electrophoresis

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† Present address: The Squibb Institute for Medical Research, New Brunswick, N. J.

cell, was used to determine microscopically the electrophoretic migration of suspended particles.

The dispersed particles to be measured were introduced into the electrophoresis cell and an electrical current was caused to pass through the cell. The time required for a specific observed particle to migrate a given distance across the cell was measured with an ocular micrometer, and the particle velocity was determined from this observation. The voltage across fixed platinum electrodes in the electrophoresis cell was recorded on a potentiometer and the electrical field strength was determined from this measurement. Experimental results were then expressed in (cm./sec.)/(v./cm.).

The zeta potential was calculated directly from this observation by using the Helmholtz-Smoluchowski (6) equation

$$\zeta = \frac{4 \pi \eta \nu}{D} \times (9 \times 10^4) \qquad \text{(Eq. 1)}$$

where ζ is the zeta potential expressed in volts, η is the viscosity of the dispersion medium in poise, D is the dielectric constant of the dispersion medium, ν is the electrophoretic mobility expressed in cm. per sec. per v. per cm. The factor (9×10^4) is included to convert from electrostatic units to the practical system of volts. It may be shown from this expression that the mobility of large microscopic particles is independent of size, shape, and orientation over a wide range. This has been confirmed experimentally by Abramson (7, 8).

The particle velocity was observed at two levels, known as the "Komagata true cataphoretic layers," of the electrophoresis cell where stationary flow occurs (9). The stationary levels were determined by observing the direction and velocity of particle migration at calculated intervals throughout the cell.

RESULTS

A plot of cell depth *vs.* electrophoretic mobility describes a parabola assuming that the velocity of electroendosmosis is the same as that of electrophoresis. Figure 1 shows the parabola for sulfamerazine and Fig. 2 shows the curve for bismuth subnitrate as determined in the electrophoresis apparatus. The two Komagata layers at 21 and 79% of the cell, respectively, may be determined from plots such as these.

With the Komagata layers selected and the potentiometer circuit balanced, the particle migration

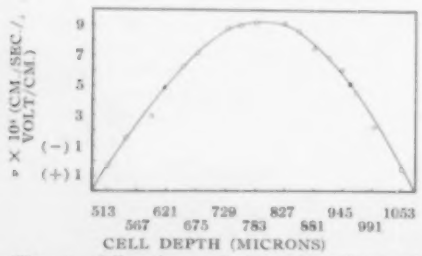

Fig. 1.—Migration velocity of sulfamerazine particles at the "Komagata true cataphoretic layers." ▲, $\nu = 4.8$ at 21% cell depth; ■, $\nu = 5.1$ at 79% cell depth.

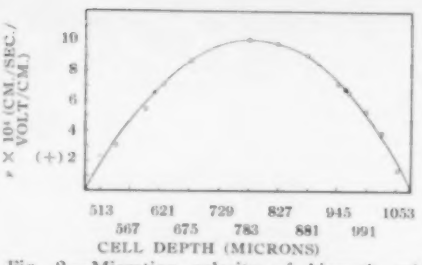

Fig. 2.—Migration velocity of bismuth subnitrate particles at the "Komagata true cataphoretic layers." ▲, $\nu = 6.6$ at 21% cell depth; ■, $\nu = 6.9$ at 79% cell depth.

was observed in the presence of flocculating electrolytes in the microscope electrophoresis cell. Initially, the electrophoretic mobility of the particles was high and they moved rapidly under the ocular micrometer. The continued addition of electrolyte produced a decrease in mobility until a critical potential was reached. At this point the van der Waals attractive forces were considered sufficient to overcome the electrostatic forces of repulsion as flocculation was observed to have occurred.

Table I lists the electrophoretic mobility of sulfamerazine in the presence of increasing concentrations of aluminum chloride. A slight excess of aluminum ions was adsorbed at one point to cause a reversal of charge effect. The sign (+ or −) refers to the charge on the particle, i. e., a (+) indicates migration to the cathode while a (−) indicates migration to the anode.

Table I.—Electrokinetic Data for Sulfamerazine U. S. P. in the Presence of Aluminum Chloride

Concn. $AlCl_3$, mM/L.	Time of Migration, sec.	ν ($\times 10^4$),[a] cm.^{2} sec.$^{-1}$ v.$^{-1}$	ζ[a] mv.	Sign of Charge	Caking Condition
0.0	32.5	4.95	63.4	−	Hard cake
0.4	40.7	3.52	45.1	−	Hard cake
1.0	63.0	2.20	28.2	−	Slight cake
4.0	73.2	1.67	21.4	−	No caking
10.0	114.1	1.09	14.0	−	No caking
40.0	190.6	0.66	8.5	−	No caking
100.0	380.4	0.32	4.1	+	No caking
400.0	420.0	0.29	3.8	+	No caking
1 M	122.8	1.05	13.5	−	Viscous

[a] Average reading of the results at the two Komagata layers.

Table II shows the electrophoretic mobility of bismuth subnitrate in the presence of increasing concentration of monobasic potassium phosphate. Above a 0.02 M concentration, phosphate ions were adsorbed in sufficient amounts to cause permanent charge reversal of the particles.

The results of the electrophoretic studies were correlated with the findings of the sedimentation-flocculation studies from previous work. They may be seen for sulfamerazine in Fig. 3 and for bismuth subnitrate in Fig. 4 where zeta potential (ζ) and the relative sedimentation height ratio (Hu/Ho) are plotted against the concentration of electrolyte added to the suspensions.

TABLE II.—ELECTROKINETIC DATA FOR BISMUTH SUBNITRATE N. F. IN THE PRESENCE OF MONOBASIC POTASSIUM PHOSPHATE

Concn. KH_2PO_4 m*M*/L.	Time of Migration, sec.	ν ($\times 10^5$)[a] cm.^{2} sec.$^{-1}$ v.$^{-1}$	ζ[a] mv.	Sign of Charge	Caking Condition
0.0	22.1	6.76	86.6	+	Hard cake
0.4	22.3	6.74	86.4	+	Hard cake
1.0	32.4	4.62	59.2	+	Hard cake
4.0	41.0	3.90	50.0	+	Slight cake
10.0	58.7	2.41	30.9	+	No caking
40.0	52.7	2.60	33.3	−	No caking
100.0	33.3	3.91	50.1	−	Soft cake
400.0	58.7	2.34	30.0	−	Soft cake
1 *M*	68.8	2.00	25.6	−	Soft cake

[a] Average reading of the results at the two Komagata layers.

DISCUSSION

Initially at point A of Figs. 3 and 4 the dispersion had a high zeta potential (negative for sulfamerazine, positive for bismuth subnitrate). The addition of the flocculating agent caused the zeta potential to decrease, and at point B, the particles were observed in the microscope of the electrophoresis apparatus to exhibit maximum flocculation. Sedimentation studies showed a low *Hu/Ho* ratio and considerable caking at point A′. The *Hu/Ho* value was maximum at point B′ and caking no longer occurred.

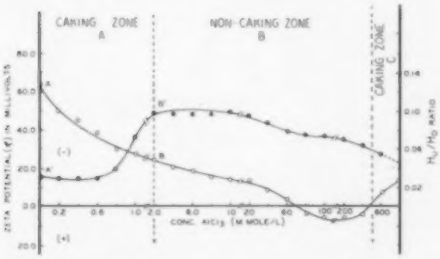

Fig. 3.—Diagram of controlled flocculation in a 2 Gm./100 ml. aqueous dispersion of sulfamerazine employing sedimentation height ratio (*Hu/Ho*) and zeta potential as indexes of caking. ●, *Hu/Ho* ratio; ○, zeta potential.

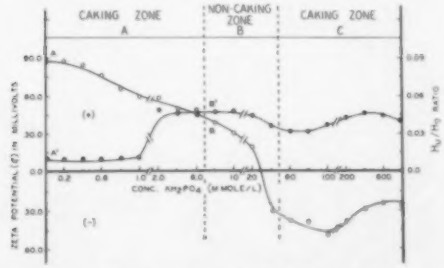

Fig. 4.—Diagram of controlled flocculation in a 2 Gm./100 ml. aqueous dispersion of bismuth subnitrate employing sedimentation height ratio (*Hu/Ho*) and zeta potential as indexes of caking. ●, *Hu/Ho* ratio; ○, zeta potential.

The high *Hu/Ho* value, together with the low zeta potential, indicated that flocculation by the added electrolyte was adequate at this point to prevent caking. Further addition of flocculating agent caused continued electrical discharge of the particles until the zeta potential dropped to zero and the particles became electrically neutral. Microscopic observation at this point showed that the particles no longer moved to the anode or cathode in the electrophoresis cell, but remained stationary in the field. On continued addition of flocculent, the electrically neutral particles assumed the charge of the flocculating agent and became increasingly positive in the case of sulfamerazine and negative in the case of bismuth subnitrate. Addition of flocculating agent far beyond the dotted boundary line X, where maximum flocculation occurred, was not needed, and high concentration of such agents are, in fact, undesirable in pharmaceutical preparations. A boundary Y could also be located beyond which the zeta potential increased sufficiently so that the particles again repelled one another and such a deflocculated system again resulted in a caked sediment. The *Hu/Ho* value began to decrease beyond the dotted boundary Y, owing to the increase of the charge density on the particles brought about by the additional concentration of flocculating ions. Accordingly, regions A and C in Figs. 3 and 4 may be considered as the caking zones, whereas region B is the noncaking zone or area of controlled flocculation, where a satisfactory product is obtained.

SUMMARY

The electrophoretic velocity in nonflocculated sulfamerazine and bismuth subnitrate suspensions was observed in a microscope electrophoresis apparatus and was found to be high, due to the predominance of a positive or negative charge on the particle. Electrical discharge of the particle by the addition of aluminum or phosphate ions, which were opposite in charge to the particle, lowered the zeta potential and brought about flocculation of the particle, thereby reducing or eliminating the tendency to cake.

It was evident in the systems under study that both the zeta potential (ζ) and the sedimentation height ratio (*Hu/Ho*) can serve as caking indexes.

The zeta potential and *Hu/Ho* ratio of aqueous dispersions may be regulated by controlling the degree of flocculation to produce stable suspensions that do not cake. It should be possible to prepare complex suspensions by treating them as individual simple suspensions, that is, by bringing each simple system into a state of controlled flocculation before completing the formulation. By observing and adjusting the electrokinetic potential after the addition of each ingredient, a complex well-flocculated suspension should be possible. It should also be possible to flocculate active ingredients with other constituents of the mixture. Various cationic amine, surfactant, and high polymer type compounds,

which obviously must first be checked for freedom from toxicity, should function well to flocculate pharmaceutical powders which are anionic in nature. Finally, it should be possible to observe and evaluate the coatings of particles by noting the changes produced in the surface charge, electrokinetic potential, and sedimentation height ratio contributed by the coating agent. Further work along these lines is to be reported in a later communication.

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Interfacial Properties of Powdered Material; Caking in Liquid Dispersions III

Adsorption Studies

By BERNARD A. HAINES, Jr.† and ALFRED N. MARTIN

Dispersed particles coated with an adsorbed film were electrophoretically observed to display surface characteristics of the adsorbent. A procedure is described for preparing suspensions with anionic or cationic characteristics by coating the particles with stearic acid or a cationic amine. By coating dispersed particles with a cationic adsorbent it was found possible to use the anionic flocculent, monobasic potassium phosphate, which was compatible with negatively-charged, viscous suspending agents, to produce a well-flocculated, noncaked suspension.

CONTROLLED FLOCCULATION of dispersed particles has been shown in previous studies to reduce or prevent caking (1). Gravity sedimentation and microscope electrophoresis were employed to observe the effects of flocculation on the particles (2). The electrokinetic or zeta potential was found to be dependent upon the kind and number of surface charges existing on a particle. Basic particle characteristics were altered upon the addition of various flocculating agents which were adsorbed onto the particle surface.

In order to simplify the method of flocculating various insoluble powders in dispersion, it is desirable to have a system composed of similarly charged particles which can be flocculated with one common agent. Such a system would be feasible if the particles of various powders were coated with a common adsorbent to produce similar surface properties. This study was conducted to determine whether or not such a procedure might be possible.

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† Present address: The Squibb Institute for Medical Research, New Brunswick, N. J.

PROCEDURE

Various fatty acid derivatives have been used to coat pharmaceutical powders. Both U. S. P. and chemically pure stearic acid was selected as the anionic fatty acid adsorbent for bismuth subnitrate and sulfamerazine particles in this study. Dodecylamine served as a cationic coating agent.

In order for optimum fatty acid adsorption to take place, the adsorbing surface must be free of extraneous materials such as adsorbed gases or vapors. For this reason the sulfamerazine employed in the study was outgassed at 10^{-6} mm. of mercury for twelve hours with subsequent heating in an oil bath at 135° for six hours. Samples to be outgassed were accurately weighed into 25-ml. ampul vials which were sealed with rubber stoppers held in place by metal crimps. The rubber stopper was pierced by a 26-gauge needle and connected by means of high pressure tubing to the vacuum system. This method of outgassing assured against contact of air with the powder. Stearic acid in dry benzene solutions was injected into the outgassed samples through the rubber stopper, thereby eliminating the possibility of moisture contamination at the solid-liquid interface.

Traces of moisture affect fatty acid adsorption in an unpredictable manner, and preliminary studies with bismuth subnitrate showed erratic results for the slightly hygroscopic powder due to bound water in the molecule. Determinations revealed that 1.23% moisture remained on the bismuth particle

after oven drying at 105° for three hours. Moisture could not be detected in the sulfamerazine powder by the Karl Fischer method. Because of the difficulty in drying bismuth subnitrate the quantitative adsorption work was continued only with the sulfamerazine powder.

The amount of fatty acid adsorbed depends upon the solvent used. Very dry benzene was employed as the solvent for the adsorbents and care was taken to preserve the solutions in air-sealed containers.

The rate of adsorption at the solid-liquid interface for sulfamerazine was determined quantitatively by measuring the adsorbent concentration before and after adsorption was completed. The quantity of stearic acid in solution initially minus the amount of stearic acid remaining in solution after adsorption was completed, presumably was the amount of stearic acid adsorbed at the powder-liquid interface. With increasing quantities of stearic acid in solution, the amount of stearic acid adsorbed per Gm. of sulfamerazine increased until a monolayer of stearic acid was adsorbed on the particle. Higher stearic acid concentrations were found to result in multilayer adsorption.

To determine the monolayer concentration, an analytical procedure was developed. Gravimetric analysis was not sufficiently sensitive to detect the small quantity of stearic acid adsorbed; however, a nonaqueous colorimetric method employing alcoholic potassium hydroxide solution proved successful. The colorimetric end point using phenolphthalein T. S. as an indicator was taken as a change from a colorless solution to the first permanent pink color. This visual end point was confirmed by potentiometric titration.

Weighed quantities of stearic acid were titrated with alcoholic potassium hydroxide solution to determine the accuracy of this method and it was found that the theoretical calculation for the stearic acid present and the actual titration results agreed within 1%.

Sulfamerazine and bismuth subnitrate were also coated with dodecylamine, a cationic adsorbent. (Dodecylamine served as a representative cationic coating agent; however, it is not suggested for use in pharmaceutical systems.) Aluminum chloride and monobasic potassium phosphate served as cationic and anionic flocculents, respectively. The effect of the flocculents on the coated particles was observed in the microscope electrophoresis apparatus as previously reported (2).

The adsorption isotherm of stearic acid on sulfamerazine powder was obtained in order to determine the concentration of adsorbent at which a monolayer was formed. Outgassed 1.5-Gm. samples of sulfamerazine, to which was added 25 ml. of a dry benzene solution containing 2.5 Gm. per 100 ml. of stearic acid, were rotated in a thermostat maintained at 25 ± 0.01°. At various time intervals samples were removed and the supernatant solution analyzed for stearic acid. It was found that adsorption was rapid and equilibrium was established after eight to ten hours. A twenty-four-hour time period was selected for the adsorption study to assure that equilibrium had been established.

RESULTS AND DISCUSSION

Apparent Adsorption Isotherm.—The amount of fatty acid adsorbed per unit weight of powder increased with increasingly low concentrations of solute until adsorption became fairly constant. A plot of adsorbed stearic acid per unit weight of sulfamerazine *vs.* stearic acid concentration exhibited a plateau region within the concentration range of about 10 to 18 Gm. per L., suggesting monolayer adsorption of stearic acid on the sulfamerazine particles. Increasing the solute concentrations resulted in a sharp rise in stearic acid adsorption and suggested the formation of a multilayer as seen in Fig. 1.

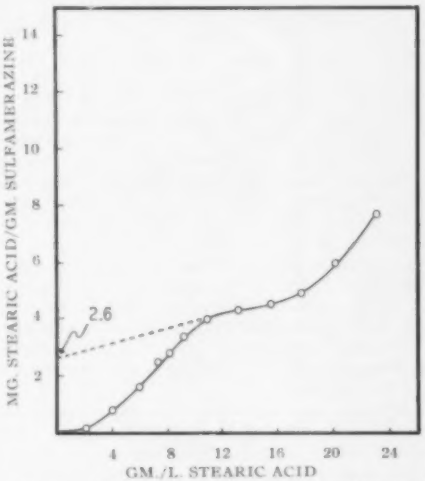

Fig. 1.—Apparent adsorption isotherm for stearic acid from benzene solution onto sulfamerazine particles.

Multilayer adsorption occurred at stearic acid concentrations greater than 18 Gm. per L. The curve corresponds to a type V adsorption isotherm described by Brunauer (3) or to a type S adsorption isotherm according to the classification of Giles and MacEwan (4) for adsorption from solution. Type V adsorption usually requires a special handling of the equation for multilayer adsorption given by Brunauer, Emmett, and Teller (5). However, the simpler form of the equation ordinarily used for type II adsorption is valid for type V adsorption when small concentrations of adsorbate, such as those employed in this study, are used.

The form of the B. E. T. equation modified by Hansen, *et al.* (6), whereby concentration terms replace the original pressure terms, was employed to describe adsorption from solution. The equation is written

$$\frac{C}{V(C_0 - C)} = \frac{1}{V_m K} + \frac{K - 1}{V_m K} \cdot \frac{C}{C_0} \quad \text{(Eq. 1)}$$

where C is the concentration of stearic acid in Gm. per L., C_0 is the saturation concentration of stearic acid, V is the number of mg. of stearic acid adsorbed per Gm. of sulfamerazine, V_m is the quantity of stearic acid required to form a monolayer on the sulfamerazine particle, and K is a constant related to the heat of adsorption and liquefaction for stearic acid. From the slope and intercept of a plot of $C/V(C_0 - C)$ *vs.* C/C_0, as shown in Fig. 2, both V_m and K

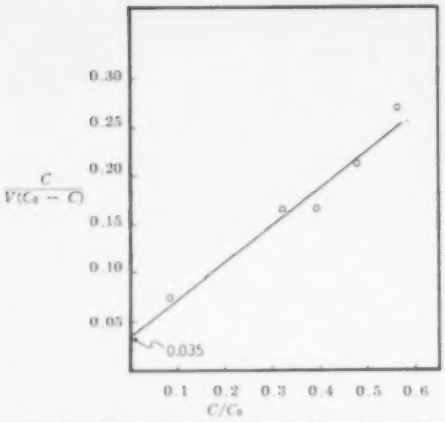

Fig. 2.—Adsorption data for stearic acid on sulfamerazine plotted according to the B. E. T. equation (5).

may be evaluated. The monolayer adsorption concentration V_m was found from such a plot to be 2.41 mg. of stearic acid per Gm. of sulfamerazine. An extrapolation to the ordinate from the plateau region of the adsorption isotherm in Fig. 1 placed the monolayer concentration at 2.60 mg. stearic acid per Gm. of sulfamerazine.

Although not required in this study, the specific surface area of the particles may be calculated according to Suito (7) from the values for V_m. It must be assumed that stearic acid is adsorbed as a monomolecular film on the sulfamerazine surface and that the particles are spheres with no pores or crevices. The cross-sectional area of the stearic acid molecule is assumed to be 21 square Ångströms, the dimension described in forming a compressed film of stearic acid at a water-air interface. The equation for obtaining the specific surface S_w is written

$$S_w = V_m N a 10^{-20}/g M \qquad \text{(Eq. 2)}$$

where S_w is the specific surface in m^2/Gm., V_m is the monolayer concentration in grams, N is Avogadro's number, a is the cross-sectional area of the adsorbate molecule, g is the sample weight in grams, and M is the molecular weight of adsorbate. The term 10^{-20} is a conversion factor for obtaining square meters from square Ångströms.

The specific surface area for sulfamerazine was calculated using the following values: V_m equals 2.41 mg./Gm., g equals 1 Gm., N equals 6.02×10^{23} molecules/mole, a equals 21 A^2, and M equals 284.5 Gm./mole. Then

$$S_w = \frac{0.00241 \times 6.02 \times 10^{23} \times 21 \times 10^{-20}}{1 \times 284.5} = 1.08\ m^2/\text{Gm.}$$

Electrophoretic Properties of Adsorbed Layers.—The electrophoretic study of powders used in suspension was reported in an earlier communication (2). The electrophoretic properties of particles containing adsorbed surface layers are found to be characteristic of the adsorbed layers as found by observing glass beads coated with gelatin in an acid medium. The negative charge of the glass beads was absent when completely coated with the gelatin solution and the nature of the surface became that of the cationic gelatin.

Anionic Adsorbents.—Bismuth subnitrate particles exhibit a positive charge when uncoated. When coated with sufficient quantities of stearic acid, an anionic adsorbent, they displayed the negative charge characteristic of stearic acid. Sulfamerazine particles displayed a negative charge both when uncoated and when coated with stearic acid. The amount of stearic acid used to coat the bismuth subnitrate and the sulfamerazine particles was arbitrarily chosen as that concentration for incomplete, mono- and multilayer coverage of sulfamerazine as previously determined. Stearic acid in concentrations of 0.5, 2, 6, 12, and 25 Gm. per L. were added to sulfamerazine and to bismuth subnitrate powders. The 0.5, 2, and 6 Gm. per L. concentrations of stearic acid were less than that required for a monolayer coverage on sulfamerazine. The 12 Gm. per L. concentration represented the apparent monolayer coverage, while the 25 Gm. per L. concentration represented multilayer coverage on sulfamerazine, as observed in Fig. 1.

Flocculation of Particles Coated with Anionic Adsorbents.—Aluminum chloride was chosen as the cationic flocculent for particles coated with an anionic adsorbent. The concentration selected was the quantity, 6 mM per L., shown to produce optimum flocculation in 20 Gm. per 1,000 ml. of aqueous sulfamerazine dispersions. The sulfamerazine results may be seen in Table I and the bismuth subnitrate results, in Table II.

The high positive zeta potential shown in Table II, columns 4 and 5, for 0.5 Gm./L. stearic acid indicates that the 0.006 M aluminum chloride flocculent contributed additional cations to the nonflocculated

Table I.—Effect of 0.006 M Aluminum Chloride Flocculation on the Electrokinetic Potential of Stearic Acid-Coated Sulfamerazine Particles

Concentration Stearic Acid, Gm./L.	ζ, mv., without Flocculent	Charge on Particle	ζ, mv., with Flocculent	Charge on Particle
0.0	63.4	−	19.6	−
0.5	47.3	−	21.1	−
2.0	52.3	−	13.6	−
6.0	47.1	−	15.0	−
12.0	39.1	−	15.5	−
25.0	41.6	−	22.1	−

Table II.—Effect of 0.006 M Aluminum Chloride Flocculation on the Electrokinetic Potential of Stearic Acid-Coated Bismuth Subnitrate Particles

Concentration Stearic Acid, Gm./L.	ζ, mv., without Flocculent	Charge on Particle	ζ, mv., with Flocculent	Charge on Particle
0.0	86.6	+	...[a]	+
0.5	33.6	+	40.3	+
2.0	55.9	−	5.1	+
6.0	59.7	−	5.5	+
12.0	62.9	−	6.4	+
25.0	65.4	−	6.3	+

[a] Bismuth subnitrate is not flocculated by aluminum ions.

and insufficiently-coated bismuth subnitrate particles.

Both positively and negatively charged particles were effectively coated when 2 Gm./L. and more stearic acid were added, as seen in Tables I and II. Their original surface characteristics were blanketed by the adsorbed ions, as shown by the negatively charged nature of the bismuth subnitrate particles coated with sufficient stearic acid (Table II). The nature of the particle surface thus determines the amount of electrolyte necessary for charge reduction.

Cationic Adsorbents.—Most hydrophilic colloids are negatively charged and when added to increase the viscosity of a medium and suspend the particles, they may interact with the heavy metal cationic flocculents, discussed in a previous communication (1), and form undesirable strings, clumps, and masses. The cationic coated materials exhibit a positive charge, and flocculents such as the phosphate ion used for these coated particles do not interact with the viscous suspending agents. These considerations led to the investigation of cationic amines as coating materials.

Low molecular weight cationic amines have an undesirable oily property that does not easily permit preparation of fine, dry coated powders. Dodecylamine, a waxy semisolid compound, was found to possess the physical-chemical properties deemed desirable for coating particles. Obviously the fatty acid amines or any other materials possessing desirable properties as flocculents or adsorbents would need to be tested clinically and accepted by the proper authorities for drug usage before being considered as pharmaceutical coating materials.

Sulfamerazine and bismuth subnitrate particles were coated with various amounts of dodecylamine, corresponding to those used for the stearic acid study, and flocculated with monobasic potassium phosphate.

Flocculation of Particles Coated with Cationic Adsorbents.—Sulfamerazine and bismuth subnitrate particles when sufficiently coated with dodecylamine from benzene solution were found to exhibit cationic characteristics. Monobasic potassium phosphate proved to be a good flocculent for the cationic adsorbent in a 6 m*M* concentration. The results may be seen in Tables III and IV.

Addition of a Suspending Agent.—The addition of a 0.25% methylcellulose solution to an anionic coated, well-flocculated sulfamerazine dispersion further reduced the electrokinetic potential to nearly zero. The resulting preparation had a uniform appearance and was composed of fine, well-dispersed floccules. Any residue on the bottom of the container could be easily redispersed by simple inversion of the container.

TABLE III.—EFFECT OF 0.006 *M* MONOBASIC POTASSIUM PHOSPHATE FLOCCULATION ON THE ELECTROKINETIC POTENTIAL OF DODECYLAMINE-COATED SULFAMERAZINE PARTICLES

Concentration Dodecylamine, Gm./L.	ζ, mv., without Flocculent	Charge on Particle	ζ, mv., with Flocculent	Charge on Particle
0.0	63.4	−	...[a]	−
0.5	24.7	−	23.4	−
2.0	12.8	+	16.4	+
6.0	27.9	+	42.0	+
12.0	45.4	+	41.4	+
25.0	50.1	+	41.0	+

[a] Sulfamerazine is not flocculated by phosphate ions.

TABLE IV.—EFFECT OF 0.006 *M* MONOBASIC POTASSIUM PHOSPHATE FLOCCULATION ON THE ELECTROKINETIC POTENTIAL OF DODECYLAMINE-COATED BISMUTH SUBNITRATE PARTICLES

Concentration Dodecylamine, Gm./L.	ζ, mv., without Flocculent	Charge on Particle	ζ, mv., with Flocculent	Charge on Particle
0.0	86.6	+	53.3	+
0.5	46.3	+	27.2	−
2.0	56.6	+	11.0	+
6.0	72.0	+	24.3	+
12.0	66.2	+	31.0	+
25.0	65.1	+	34.7	+

The negative charge shown in Table IV, column 5, for 0.5 Gm./L. dodecylamine indicates that the 0.006 *M* monobasic potassium phosphate flocculent was present in a sufficient amount to reverse the zeta potential from a low positive value for the nonflocculated particles to a low negative value for the flocculated particles. At higher concentrations of dodecylamine, the anionic flocculating agent can no longer reverse the charge from positive to negative.

SUMMARY

Sulfamerazine and bismuth subnitrate particles coated with anionic and cationic agents were found to possess properties similar to the coating agent. The construction of an adsorption isotherm assisted in determining the amount of adsorbed material necessary to provide desired coating characteristics; electrokinetic observations provided a measure of dispersion stability.

Either negatively charged sulfamerazine or positively charged bismuth subnitrate particles, coated with a cationic coating agent, can then be flocculated partially to render the suspension free from caking. The dispersion can then be stabilized against sedimentation by the addition of a suitable hydrophilic colloid.

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Some Cardiovascular Effects of Trimethobenzamide

By DON N. BENSLAY and ROB S. McCUTCHEON

A cardiovascular investigation of trimethobenzamide was made in regard to (*a*) the mechanism of its hypotensive properties and (*b*) any antiarrhythmic effects. Under (*a*) blood pressure studies were carried out while using autonomic agents in conjunction with trimethobenzamide on anesthetized mongrel dogs. Of the autonomic agents tried, only ergotamine effectively prevented the hypotensive effects. Under (*b*) electrocardiograms were recorded on mongrel dogs in which cardiac arrhythmias had been induced. These studies indicate that the drug serves to prolong the time of previously induced auricular fibrillation and has little effect on prevention of arrhythmias. Some evidence indicated that trimethobenzamide protects the heart from ventricular fibrillation.

TRIMETHOBENZAMIDE is the generic name of a compound chemically designated as 4-(2-dimethylaminoethoxy)-N-(3,4,5-trimethoxybenzoyl) benzylamine hydrochloride (1). It was first synthesized in the chemical research laboratories of Hoffmann-La Roche, Inc. It is now considered a specific antiemetic drug and is marketed as such.

Many agents have been used in the past as antiemetics but most can be classed in one of three categories: antihistamines, reserpine, and phenothiazine derivatives. Trimethobenzamide belongs to none of these groups but incorporates some of the chemical structure of each. It is largely free of the side effects commonly associated with the above three groups of drugs. It has a transient blood pressure lowering effect and produces some indications of central excitement (1). Because of the cardiovascular properties shown by trimethobenzamide and because of these properties seen in other members of the antiemetic groups it seemed of importance to make a cardiovascular study of this substance.

EXPERIMENTAL

Blood Pressure Studies.—Blood pressure studies already reported (1) were repeated for purposes of confirmation. Also, an attempt was made to determine the way in which the drug was lowering blood pressure. Experiments were conducted in six groups: (*a*) The experimental agent, in which trimethobenzamide was used alone as an i. v. injection in a dosage of 4 mg./Kg. Effects on blood pressure were observed. (*b*) Antihistaminic agents, in which chlorpromazine[1] or diphenhydramine were given in conjunction with the administration of trimethobenzamide. (*c*) Adrenergic, in which levarterenol was used in conjunction with trimethobenzamide. (*d*) Sympatholytic agents in which phenoxybenzamine, dichloroisoproterenol, and ergotamine were used in conjunction with trimethobenzamide. (*e*) Cholinergic agent in which acetylcholine was used in conjuntion with trimethobenzamide. (*f*) A parasympatholytic agent, atropine, with trimethobenzamide. For details of dosage and order of injection of the various combinations, see Table I.

Twenty-one mongrel dogs of either sex weighing between 10 and 25 Kg. with an average weight of 16 Kg. were used in this study. Each was anesthetized with pentobarbital sodium solution, 30 mg./Kg., administered intraperitoneally. Supplemental doses were given if needed. Blood pressure was obtained primarily with a mercury manometer using sodium citrate 4% in the system. Recordings were made on a smoked drum of an electric kymograph throughout the entire experiment. In some cases a Sanborn recorder was utilized in conjunction with a physiological pressure transducer to record blood pressures. The right common carotid artery was exposed through an incision and cannulated with a glass arterial cannula. Single doses of drugs were administered rapidly by injection via the femoral vein (five to ten seconds). When two drugs were administered simultaneously, one was by constant infusion through an indwelling catheter in the femoral vein using a Harvard constant volume infusion pump. The rate of infusion was determined usually by the blood pressure response to the drug. Heparin sodium[2] was administered in all experiments as an anticoagulant in a dosage of 2 mg./Kg.

Cardiac Studies.—The purpose of this study was to determine the effect of trimethobenzamide in relation to cardiac arrhythmias in the dog.

Ten mongrel dogs of either sex weighing between 12 and 22 Kg. were used in this study. Each was anesthetized with pentobarbital sodium solution, 30 mg./Kg., administered intraperitoneally. Supplemental doses were given if needed. The electrocardiogram was recorded using a Sanborn model 150 four channel recorder. The standard limb leads (2) were employed, the lead wires being connected to the animal by placing needles under the skin. In all experiments a Sanborn Viso Scope oscilloscope was employed so that one lead could be viewed continually. In some experiments the blood pressure was recorded simultaneously using a Sanborn physiological pressure transducer connected to the right common carotid or the femoral artery

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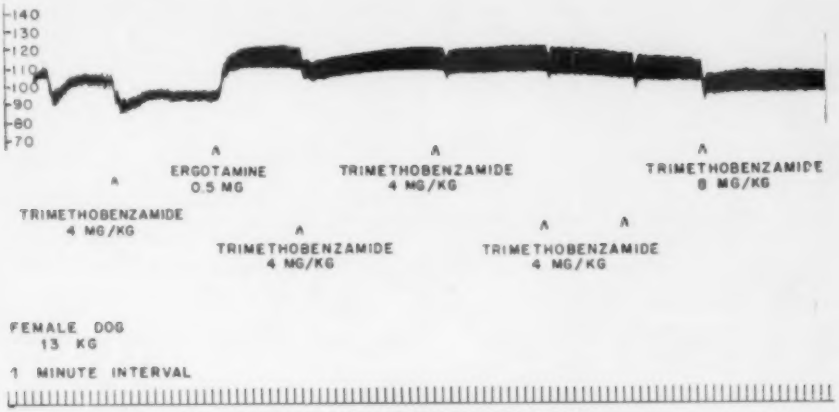
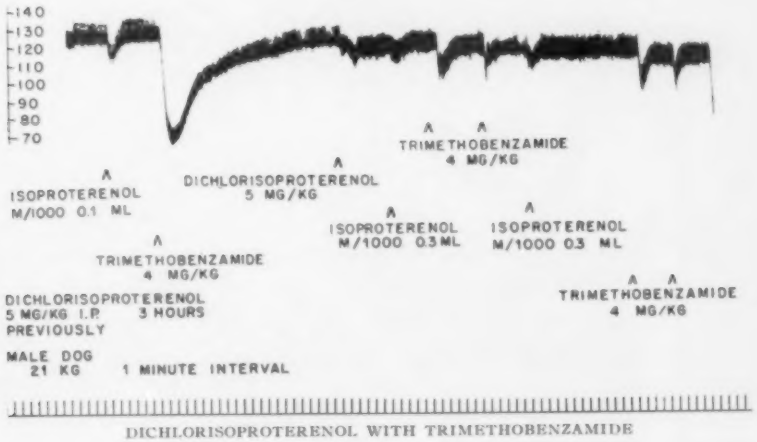
[1] Classed for convenience with antihistamines.

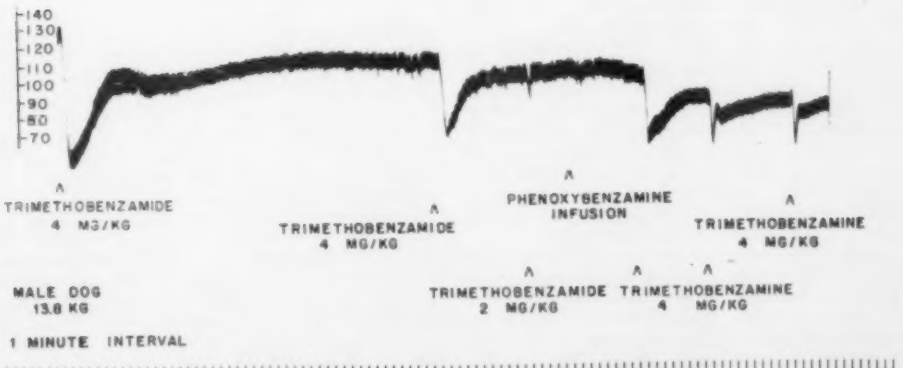
[2] Lilly ampul No. 405.



ERGOTAMINE TRIMETHOBENZAMIDE



DICHLORISOPROTERENOL WITH TRIMETHOBENZAMIDE



PHENOXYBENZAMINE WITH TRIMETHOBENZAMIDE

Fig. 1. Some drug combinations and blood pressure changes.

TABLE I.—MEAN ARTERIAL BLOOD PRESSURE CHANGES PRODUCED BY EXPERIMENTAL AGENTS

Drug Combination	Dose, i. v.[a]	B. P., mm. Hg	N[b]	S. D.[c]
Trimethobenzamide[d]	4 mg./Kg.	−34.67	15	±20.83
Chlorpromazine	1 mg./Kg.	−33.75	4	±2.50
Chlorpromazine after trimethobenzamide	1 mg./Kg.	−37.50	2	±2.50
Trimethobenzamide after diphenhydramine	30 mg./Kg.	−15.00	2	±7.18
Trimethobenzamide during levarterenol infusion	10 mcg./Kg./min.	−37.50	4	±2.50
Trimethobenzamide after ergotamine	31 mcg./Kg.	−8.55	11	±3.27
Trimethobenzamide during phenoxybenzamine infusion	30 mcg./Kg./min.	−26.50	10	±5.79
Acetylcholine during trimethobenzamide infusion	65 mcg./Kg.	−70.00	2	±0
Trimethobenzamide after atropine	1 mg./Kg.	−27.00	5	±2.79

[a] The doses given in the above table pertain to the drug other than trimethobenzamide used in the combination.
[b] Number of observations.
[c] Standard deviation, S. D. = $\sqrt{\frac{\Sigma(y-\bar{y})^2}{N-1}}$
[d] The dose of trimethobenzamide was always 4 mg./Kg., i. v., and when administered by i. v. infusion, it was approximately 0.7 mg./Kg./min.

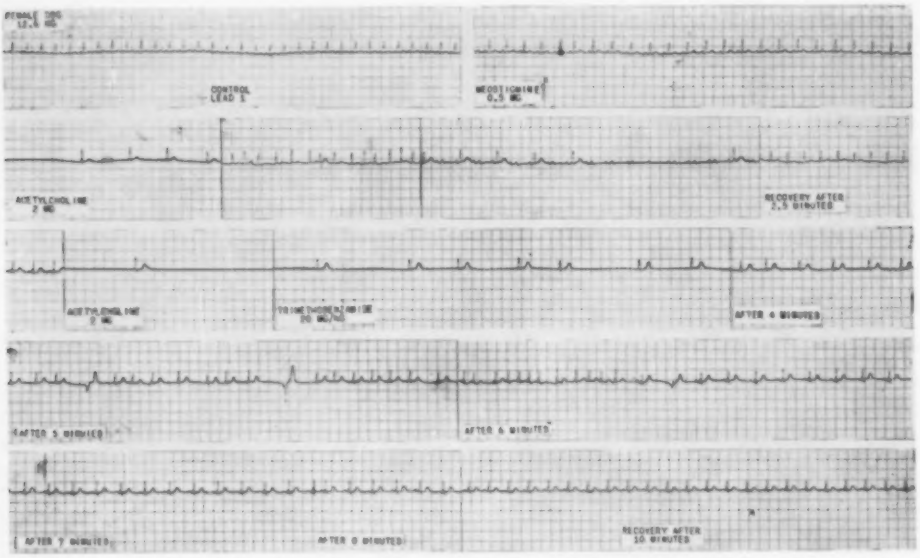


Fig. 2.—Electrocardiogram showing prolonged arrhythmia.

and recorded on one channel of the recorder. When needed, a Harvard respiration pump model No. 607 was employed to support respiration. The pump, operating through a tracheal cannula, was set for 20 respirations per minute, and the volume delivered varied depending upon the size of the animal. The dogs were positioned on the dorsal side ordinarily, and on the ventral side when acetylcholine was used. Drugs were administered directly into either the saphenous or the femoral vein.

Defibrillation was attempted at times by administering a shock lasting one-half second with 110 volts of alternating electric current (3, 4).

After consideration of methods set forth in the literature it was decided that two methods of inducing cardiac arrhythmias could well be used in the present studies. The first method consisted of injection of acetylcholine after use of an anticholinesterase agent, prostigmine, 0.5 mg. per animal (5, 6). The second consisted of small doses of epinephrine 2 mcg./Kg. during inhalation of chloroform (7).

RESULTS AND DISCUSSION

Table I shows the various drug combinations used, the order and dosage of injection, and the effects of these drug combinations on mean arterial blood pressure.

It is clear from the table that the results with trimethobenzamide alone confirm the results reported by Schallek (1). When trimethobenzamide is given in combination with chlorpromazine, little difference can be noted in overall effect and it can be said that neither drug is altering the action of the other. Diphenhydramine may be preventing the typical blood pressure fall to be seen with trimethobenzamide alone. It is also of interest that the blood pressure fall produced by trimethobenzamide was not retarded in any way by levarterenol. In a single trial with epinephrine the same result was noted. The blood pressure fall after ergotamine was apparently partially blocked. The average fall in these circumstances is only about one-fourth of that produced by trimethobenzamide alone. It is question-

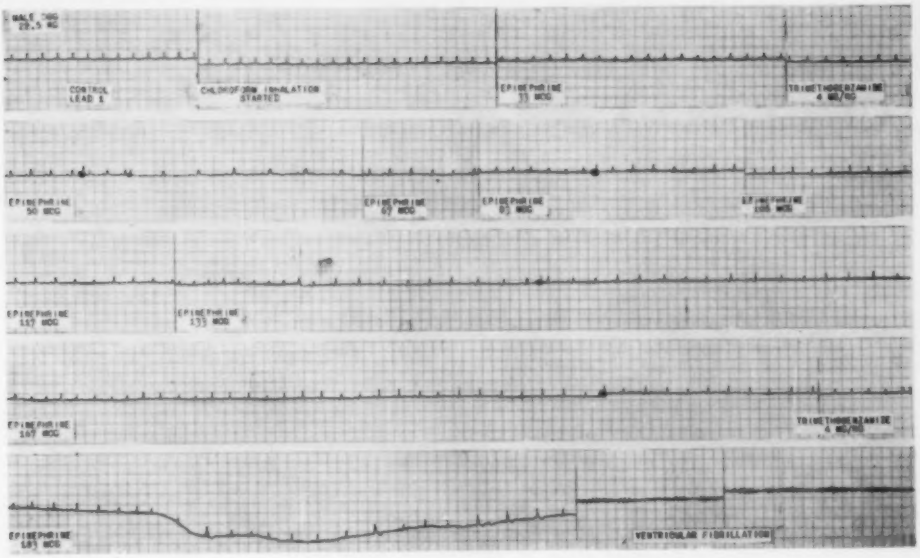


Fig. 3.—Electrocardiogram showing increased dose of epinephrine.

able whether this blocking action is due to the sympatholytic properties of ergotamine. It may be due to its strong vasoconstricting effects on the peripheral blood vessels. This is indicated because of the lack of any strong blocking action shown by the other sympatholytic agents used in the study. Phenoxybenzamine decreased the amount of blood pressure fall produced by trimethobenzamide slightly. Since this action is similar to that of ergotamine it may be an indication of a partial block produced by these agents. See Fig. 1.

The effect of the combination of acetylcholine with trimethobenzamide appears to be purely an additive one and of no significance.

The cardiac studies showed that trimethobenzamide protects the animal against ventricular fibrillation but causes a prolonged auricular fibrillation after it has been induced, as shown by the electrocardiogram in Fig. 2.

Experiments on one animal, in which an auricular fibrillation had been induced by the epinephrine during chloroform inhalation method, indicated that after an injection of trimethobenzamide, the control dose of epinephrine that would usually precipitate a ventricular fibrillation could be far exceeded. See Fig. 3. When trimethobenzamide was injected before the induction of an arrhythmia by either method used in the study, it was noted that the injection in no way prevented the onset of the arrhythmia subsequently induced. It was shown also in the experiments, that the injection of trimethobenzamide during the course of a previously induced arrhythmia (by either method) only served to prolong the time of the arrhythmia. The prolongation of time over the control arrhythmia varied with size and individuality of the animal used.

SUMMARY

1. The blood pressure effects of trimethobenzamide have been confirmed.

2. The effects of combinations of various autonomic drugs with trimethobenzamide indicate that the blood pressure effects are probably not mediated through an autonomic mechanism.

3. Auricular fibrillations were prolonged by the action of trimethobenzamide but the heart was protected against ventricular fibrillation.

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Estimation of Mercurial-Protein Binding by a Polarographic Method

By SISTER M. LAWRENCE O'DONNELL, ANNA MAE SEBULAK, and CORNELIUS W. KREKE

The polarographic method of Tadao Hata has been studied as a means of estimating both primary and secondary binding of organic mercurials on proteins. *p*-Chloromercuric benzoate and ethyl mercuric hydroxide were found to bind strongly with native crystalline egg albumin in a molar ratio of 2:1 while phenylmercuric hydroxide was found to exhibit weak sulfhydryl binding and a large amount of secondary binding. Urea, 3 *M*, eliminated the secondary binding of the PMOH and increased the primary binding to the level of the PCMB and EMOH. Stronger denaturants such as 6 *M* urea and guanidine increased the sulfhydryl binding of the PCMB but decreased the binding of the PMOH. The results are interpreted in terms of two mechanisms of binding, whole molecule binding and dissociation, with whole molecule binding playing the primary role in mercaptide formation and dissociation accounting for the secondary binding of the PMOH.

THE ESTIMATION of the binding of organic mercurials on proteins has been done primarily to ascertain the presence of reactive sulfhydryl groups. The methods employed include chemical and amperometric titrations and also spectographic estimations, with *p*-chloromercuribenzoate selected, for the most part, as the test reagent. These methods, while successful in the study of the primary binding or sulfhydryl binding of the mercurial, do not lend themselves to the study of the total binding sites, including those referred to as secondary. The specificities of these methods either exclude the possibility of estimating secondary binding, as in the case of chemical or spectographic determinations or consider secondary binding as an interference in the estimation of primary binding, as in the case of amperometric titrations.

Among organic mercurials of widely different structure, selective biological action, and different toxicities, it seems essential that the total protein binding be considered if there be hope of relating structure to biological action or hope of understanding the mode of their diverse actions. The polarographic method suggested by Hata (1) in 1951 offers the possibility of a differentiation of, and an estimation of, the total primary and secondary binding of organic mercurials on proteins. In this method, the depression of the polarographic wave height of the mercurial in the presence of increasing concentrations of protein is determined, and the amount of mercurial bound to the protein estimated from a standard concentration curve of the mercurial. The usefulness of this method in estimating sulfhydryl groups was investigated by Hata (2) and Ito and Suzuki (3).

This present paper is a report on the evaluation of this method, in which the binding of three mercurials on crystalline egg albumin was studied. Egg albumin was chosen as the test protein because the number of reactive sulfhydryl groups in both the native and denatured protein has been determined by a number of methods (4–6). *p*-Chloromercuric benzoic acid (PCMB), phenylmercuric hydroxide (PMOH), and ethyl mercuric hydroxide (EMOH) were chosen as the mercurials because they show well-defined polarographic waves, have similarities in their structure, and have been studied as biological inhibitors. Also, as mentioned before, PCMB has been used as a standard test reagent in a number of sulfhydryl studies.

METHODS AND MATERIALS

The polarographic method of Hata is based on the fact that the reaction of proteins with mercurials renders the bound mercurial polarographically inactive, with a resulting loss in wave height. This loss in wave height is directly proportional to the amount of mercurial bound since it was found in these laboratories, and also by others, that the wave heights of the polarograms were directly proportional to the concentration of mercurials between a concentration of 10^{-5} and 10^{-4} *M*.

The polarograms were made using an American Optical recording polarograph model G-I. On this instrument, microamperes = millimeters × sensitivity/100. The potentials were measured against the saturated calomel electrode. A drop time (t) of 3–5 sec./drop, under a pressure of 45 cm. of mercury, was maintained as standard procedure. The supporting electrolyte was 0.1 *M* ammonium chloride-ammonium hydroxide buffer pH 7.4.

Wave height was measured on the plateau of the first wave of the mercurial at a voltage of −0.5 v. The diffusion current (i_d) of the mercurials alone in

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buffer solution was found to vary from 35 to 40 ($S/100$) μa. for the PMOH,[1] 27 to 31 ($S/100$) for the PCMB,[2] and 47 to 52 ($S/100$) for the EMOH.[3] In the 6 M urea solution there was only a slight lowering of this height from 24.5 to 29.5 ($S/100$) for the PMOH and 25 to 28 ($S/100$) for the PCMB. The diffusion current of the buffer alone varied from 16 to 18 ($S/100$) and, with the 6 M urea, 10 to 15 ($S/100$) μa. Since these values supplied the upper and lower limits for the determination of the moles of mercurial bound, they were determined each day with each set of experiments. In all the experiments, the polarograph was set at $S = 2$.

Stock solutions of the mercurials were made at a concentration of 2×10^{-4} M and 50 ml. of these solutions were diluted with 50 ml. of 0.2 M buffer which contained the albumin and urea as needed. These solutions were allowed to stand for twenty minutes, then deoxygenated with oxygen-free nitrogen for twenty minutes, then stoppered and allowed to stand twenty minutes more to attain a temperature of 25–27° before analyzing on the polarograph.

Preliminary experiments were conducted using soluble egg albumin (Fisher Scientific Co.) and then were confirmed using crystalline albumin (Nutritional Biochemicals Corp.). During the course of these experiments no differences were noted in the binding of the mercurials on these two albumins outside the normal variations of the control experiments.

RESULTS

The binding of egg albumin with PCMB by this polarographic procedure is shown in Fig. 1. It will be noted that only negligible binding of the mercurial was observed with a concentration of 1×10^{-6} M albumin. At lower concentrations of albumin no binding was observed. The suppression of the wave height was complete at a concentration of 5×10^{-6} M albumin, indicating a binding ratio of PCMB to albumin of 2:1. This molar binding ratio varied only slightly as the concentration of albumin was increased, being 1.9:1 at 2×10^{-6} albumin, 1.8:1 at 3×10^{-6} M, and 2.0:1 at 5×10^{-6} M as indicated in Fig. 1 at the experimental points. These results are approximately in agreement with those of MacDonnell, *et al.* (5), who found a 2.85 to 2.95 equivalent per mole of SH in the native albumin using the titration procedure at pH 5.3 and lower values at pH 7.0. Also, they obtained a binding of 2.18 equivalent per mole protein by an analysis of the mercurated protein. The results are also in good agreement with the work of Anson (7, 8) who found that iodoacetamide blocked 40% of the SH groups in native albumin. This is a binding ratio of 1.9 equivalents per mole, assuming Anson's estimation of the total sulfhydryl content of 4.75 equivalents per mole in the denatured albumin to be correct. Amperometric titration, using the dropping mercury electrode, also gave 2:1 binding of the PCMB, using the same chloride buffer.

A complex binding curve was obtained when PMOH was studied polarographically with egg albumin. Very low concentration of the albumin, even as low as 1×10^{-7}, showed appreciable suppression of the wave height of PMOH but the molar ratio of binding varied as the concentration of albumin increased, giving rise to an S-shaped curve, as shown in Fig. 2. A somewhat constant ratio of binding was obtained between the concentrations of 3×10^{-6} and 5×10^{-6} M protein and this was assumed to be sulfhydryl binding, since it seemed to imitate PCMB binding in comparable concentrations. It is referred to as primary binding in the graph (1.09–1.36 equivalents per mole). Above this concentration of protein the binding of PMOH increased slightly up to a concentration of 1×10^{-4} M albumin. The fact that even high concentration of albumin (1×10^{-4} M) was unable to suppress the PMOH wave completely suggests a lower affinity of this mercurial for the protein as compared to the PCMB. The suppression of the wave height by low concentration of protein (3×10^{-6} M) was assumed to be due to secondary binding, that is, binding of groups in addition to the sulfhydryl. The suggestion by Hata and Matsushita (2) that small amounts of suppression may be due to polarographic interference by the protein, e. g., by change in viscosity, does not seem to be applicable here since no protein effects of this kind were noticabie when PCMB was studied or when the protein was denatured. The other suggestion of this author that a slight binding effect may be due to non-

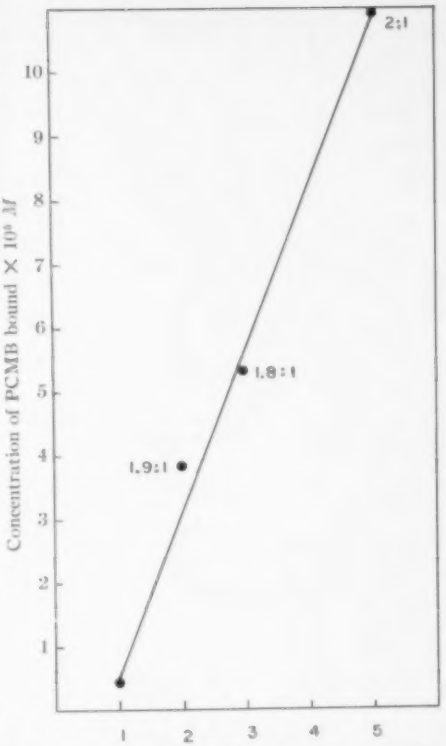

Fig. 1.—Binding of PCMB on native egg albumin. Molar ratios PCMB to albumin given at experimental points.

[1] Supplied by F. W. Berk and Co. and recrystallized from water, m. p. 224–227°.
[2] Bios Laboratory Product.
[3] Obtained from Delta Chemical Works.

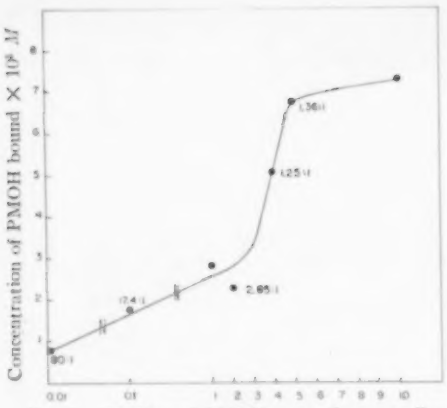

Fig. 2.—Binding of PMOH on native egg albumin. Molar ratios PMOH to albumin given at experimental points.

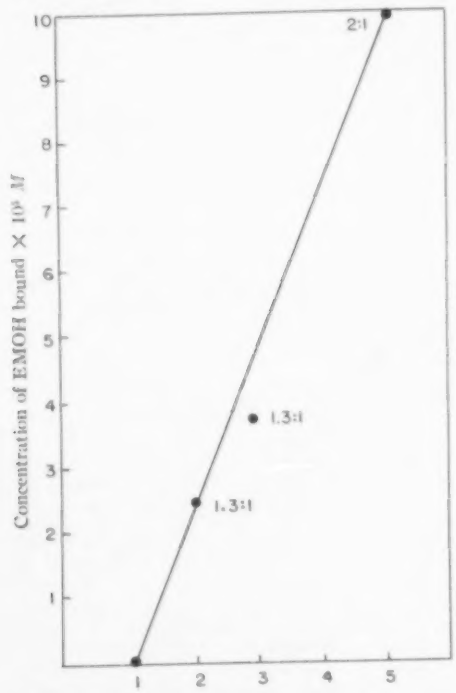

Fig. 3.—Binding of EMOH on native egg albumin. Molar ratios of EMOH given at experimental points.

specific reaction or adsorption is applicable only to PMOH binding. The nature of this secondary binding is unknown and whether it is an influence on the primary binding cannot be suggested. These findings do suggest, however, that the SH groups may be partly masked for the PMOH.

The difference in the binding of the two mercurials is not due to a difference in the anion moiety of the molecule. Under the conditions of these experiments both mercurials should be present to a large extent as the undissociated chloride. Unpublished data of Benesch and Benesch (9) show that the affinity of hydroxyl ions for PCMB is greater than that of chloride ions by a factor of about 10^4. Assuming an equal affinity for the PMOH and EMOH, the mercurials should be present as chloride under the conditions of our experiments (ammonium chloride-ammonium hydroxide buffer) in a ratio of approximately 50:1 as compared to hydroxide.

Some confirmation of this assumption is presented by the fact that the binding of EMOH on the native egg albumin is similar to that of PCMB. The results of this study are shown in Fig. 3. The EMOH shows no evidence of secondary binding and appears to have a great affinity for the sulfhydryl groups in that it is completely bound to the protein at a concentration of 5×10^{-5} *M* albumin, giving a molar binding ratio of 2:1.

These experiments were repeated using denatured albumin in order to test the hypothesis that these denaturing agents would unmask any unavailable SH groups. The protein was denatured by allowing it to remain in contact with increasing concentrations of urea up to 6 *M* for one hour, that is, through the period of standard treatment. In some experiments an attempt was made to obtain additional denaturation with 6 *M* guanidine and by boiling the albumin in the 6 *M* urea or 6 *M* guanidine for fifteen minutes prior to deaeration.

In Fig. 4 is shown the results of a series of experiments in which PMOH was bound on 3 *M* urea-denatured albumin. In the region of primary binding (3×10^{-5} to 1×10^{-4} *M* albumin) there was an increase in the binding of PMOH to a ratio similar to that of the PCMB on the native protein (2:1). There was also an apparent increased affinity of the PMOH for the protein, being bound 92% with a concentration of 5×10^{-5} *M* albumin. Also the secondary binding of the PMOH was eliminated.

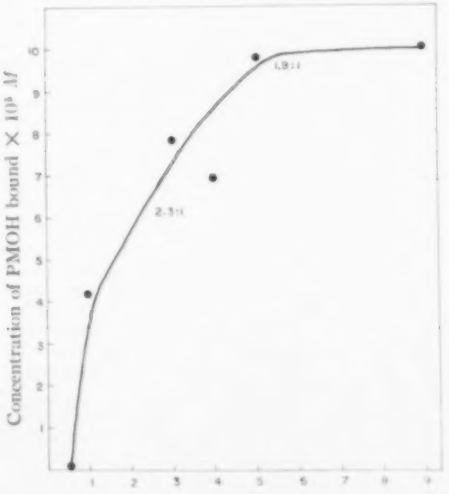

Fig. 4.—Binding of PMOH on 3 *M* urea-denatured albumin. Molar ratios PMOH to albumin given at experimental points.

Apparently the 3 *M* urea was able to unmask the SH groups for the PMOH but it did not change the amount of the binding of PCMB, indicating that its concentration was too low to disrupt the protein molecule.

In Fig. 5 are shown the results of a series of experiments in which PMOH was bound on 6 *M* urea-denatured albumin. In the region of primary binding (3×10^{-5} to 1×10^{-4} *M* albumin) the ratio of molar binding of PMOH to albumin was decreased as compared to native protein being 1:4 at a concentration of 4×10^{-5} *M* albumin and increasing to 1:1.2 at a concentration of 1×10^{-4} *M* albumin.

The urea, however, did increase the binding capacity of the protein for PCMB as would be expected from its well-known ability to unfold proteins and release masked SH groups. These results are shown in Fig. 6. The depression of the wave is appreciable in the concentration of 1×10^{-5} *M*, indicating an approximate binding of 2.8:1. This ratio was maintained to a concentration of 3×10^{-5} *M* protein where 8.24×10^{-5} *M* PCMB was bound. This binding theoretically should reach completion at a concentration of 3.7×10^{-5} *M* albumin as shown by extrapolating the line beyond 3×10^{-5} *M* protein. This value of molar binding for PCMB is lower than that reported by a number of authors (see Putnam's review) (4) where values of the equivalents of SH groups per mole of egg albumin range from 3.42 to 3.83; however, it is in partial agreement with MacDonnell (5) on the basis of the observation that the binding of PCMB is lower at higher pH. The significant poiat here is that the urea released unavailable sulfhydryl for the PCMB but not for the PMOH.

Attempts to release additional thiol groups for PMOH binding with more drastic denaturation of the protein using 6 *M* guanidine hydrochloride and boiling in 6 *M* urea failed. The results of these experiments are given in Tables I and II. It will be noted again that the binding ratio of PMOH to albumin is actually lower with these denatured proteins than it was with the native protein itself in the primary binding range. This unexpected effect of 6 *M* urea was also observed before by Jensen in his study of the binding of silver ions on bovine plasma albumin (10).

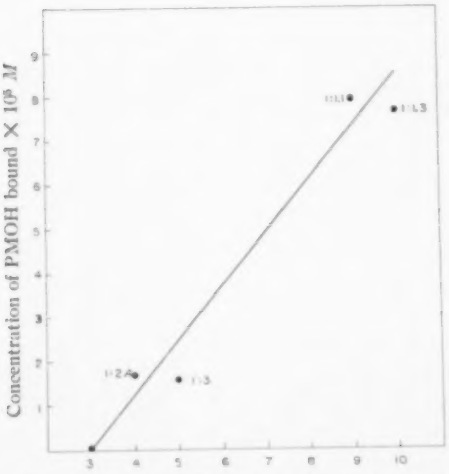

Fig. 5.—Binding of PMOH on 6 *M* urea-denatured albumin. Molar ratios PMOH to albumin given at experimental points.

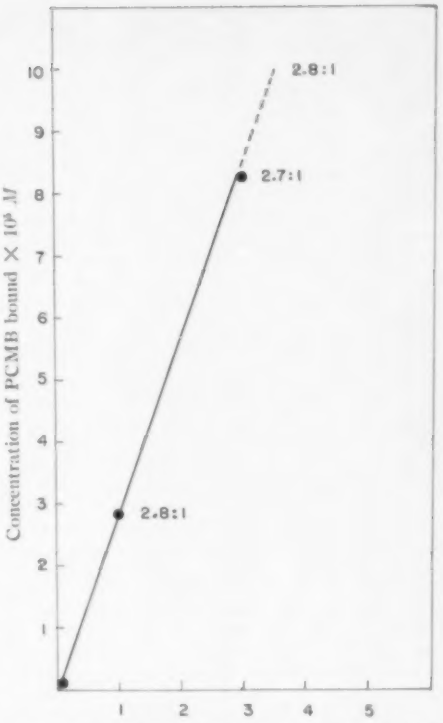

Fig. 6.—Binding of PCMB on 6 *M* urea-denatured albumin. Molar ratios PCMB to albumin given at experimental points.

TABLE I.—BINDING OF PMOH ON HEAT UREA-DENATURED ALBUMIN

Moles of PMOH Bound per L. Solution × 10^5	Moles of Albumin Added per L. Solution × 10^5	Molar Ratio, PMOH:Albumin
1.2	2	0.6:1
2	3	0.66:1
2.3	4	0.57:1
2.6	5	0.52:1
4.3	6	0.72:1
4.2	7	0.60:1
6.7	8	0.84:1
10.0	9	1.1:1

TABLE II.—BINDING OF PMOH ON GUANIDINE-DENATURED ALBUMIN

Moles of PMOH Bound per L. Solution × 10^5	Moles of Albumin Added per L. Solution × 10^5	Molar Ratio, PMOH:Albumin
3.1	3	1.03:1
3.3	4	0.81:1
6.25	10	0.62:1

DISCUSSION

From these results it is evident that the polarographic method of Hata can produce valuable information concerning the binding of mercurials on proteins. The method should be applicable to all organic mercurials provided they exhibit distinct polarographic waves and the relation of wave height to concentration of mercurial is determined. Also, the method should be applicable to other proteins, provided that the protein is pure and has no characteristic wave. The method has the distinct advantage in that it allows conditions for a study of the secondary binding of the mercurial, that is, conditions under which there are large excesses of mercurial.

The unusual binding of the PMOH exhibited both by a weaker primary binding and a large amount of secondary binding must be due to a difference in the organic part of the molecule since, as was shown before, the mercurials should be present largely as the chloride species under the conditions of these experiments. This difference in binding may be due to a relatively large polarizability of the phenyl group as compared to the ethyl group in the one case and the phenyl with a *para* carboxyl in the other. It is conceivable that this greater polarizability would result in a greater electron density on the mercury in the case of PMOH (or really phenylmercuric chloride, PMCl, in the buffer solution) and should encourage a greater dissociation of the PMCl. With this notion, it is easy to see why the PMOH (or better PMCl) would show a lesser affinity for the sulfhydryl groups of the albumin. Of course, the two sulfhydryl groups that bind so well with the PCMB and EMOH must also be partially masked for the PMOH since, with 3 *M* urea, they became available for reaction. Apparently this masking in some way involves weak hydrogen bonding, since higher concentrations of urea were necessary to uncover additional sulfhydryl groups.

The secondary binding of the PMOH apparently does not involve hydrogen bonding at the large number of binding sites, even though this binding is completely eliminated by 3 *M* urea. In amperometric titrations, using the dropping mercury electrode, it was found that the PMOH actually binds with the urea with a high diffusion current at −0.5 v. for the combination. It seems reasonable that the secondary binding of the PMOH (or PMCl) may be due to the adsorption of a PM^+ ion on the protein, if the polarizability of the phenyl group is great enough to encourage an appreciable amount of this dissociation. The elimination of the secondary binding by the urea then appears to be due to an adsorption of the PM^+ ion on the urea (3 *M*) without appreciable change in wave height of the mercurial.

The results of this work suggest strongly that binding of organic mercurials on proteins is not dependent on a dissociation mechanism alone, but also may follow the path of the whole molecule binding, producing an intermediate complex as shown in the formula

$$\begin{array}{l} \text{protein}\text{—}\overset{\displaystyle |}{\underset{\displaystyle |}{\text{S}}}\text{—H} \\ \qquad\quad \text{P—M—Cl} \end{array}$$

If dissociation were the only mechanism of mercaptide formation, then one would expect the PMOH (PMCl in solution) to be at least as active in SH binding (or probably more so because of the greater polarizability of the phenyl group) as the PCMB and EMOH (EMCl in solution). This is clearly not the case. Also, if dissociation were the primary mechanism, one might expect greater binding of mercurials at higher pH, where the SH would be more highly ionized. This is not true according to a number of investigations, including those of MacDonnell (5).

To throw more light on the mode of binding and the basis of biological selectivity, a similar study of a number of other mercurials of different structures and of different biological action is now in progress.

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Local Anesthetics III

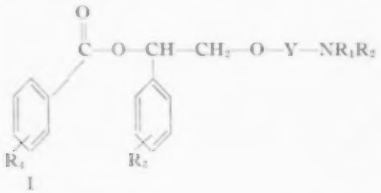
Dialkylaminoalkoxyphenylethanol Esters

By SEYMOUR L. SHAPIRO, HAROLD SOLOWAY, HARRIS J. SHAPIRO, and LOUIS FREEDMAN

A series of "procaine analogs" (I) was prepared by reaction of dialkylaminoalkoxyphenylethanols with aromatic acid chlorides. Upon evaluation as anesthetics, selected compounds showed considerably more activity than procaine.

IN THE "PROCAINE" type of local anesthetics (1), lengthening of the alkylene chain separating the tertiary amino group and the ester group increases anesthetic activity as well as toxicity. These conclusions are substantiated in the recent work from other laboratories (2, 3).

Extension of our explorations (4) to compounds of type I was indicated in the hope of increasing anesthetic potency with no accompanying increase in toxicity.



I

R_1, R_2 = lower alkyl
$R_1 + R_2$ = lower alkylene
Y = ethylene, propylene, and trimethylene
R_3 = hydrogen and methyl
R_4 = hydrogen, alkyl, alkoxy, and halogen

For the reactant alcohols, the amino alcohol, R_1,R_2N-Y-OH (in excess) was condensed under alkoxide catalysis (5) with styrene oxide. This reaction, while affording the desired dialkylaminoalkoxyphenylethanols (Table I), was clearly not as straightforward as the condensation of secondary amines with stryene oxide (6). Under these reaction conditions the product formed is largely the secondary alcohol (5, 7, 8). A variety of nonbasic side products (9–12), including styrene glycol, was obtained in some of the preparations.

Alternatively, the substituted ethanols of Table I were preparable by palladium reduction of ω-(dialkylaminoalkoxy)-acetophenones which, in turn, were obtained from phenacyl halide and the reactant dialkylaminoethanol (see Experimental).

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Treatment of the substituted ethanol with the acid chloride in benzene or in acetonitrile gave the ester I (Table II) isolated as the hydrochloride, or preferably, as the free base by distillation.

The pharmacological data (Table III) reflect that the compounds of Table II are more potent anesthetics, although slightly more toxic, than the esters of our previous series (4).

The most active $—NR_1R_2$ variant was pyrrolidino (compound 9). Variation of R_3 as hydrogen or methyl (compound 1 *vs.* 2) indicates superiority of hydrogen. The R_4 variant is desirably retained as hydrogen, although R_4 = *p*-CH_3O— (compound 5) combined high toxicity with anesthetic effectiveness.

With the ethanols of Table I, the antitremorine effect was noteworthy. Employing the procedure of Shapiro, et *al.* (4), the following observations were made: Compound No./$LD_{min.}$ mg./Kg./tremorine ED_{50} mg./Kg., respectively: 4/>1,000/32; 5/750/140; 9/750/90. On the other hand, compound 7 afforded moderate lasting hypotension (13).

Also of interest was the toxicity ($LD_{min.}$ of 100 mg./Kg.) of the dibutylamino derivative (compound 8) whereas the other amino alcohols had $LD_{min.}$ levels in the range 750 to >1,000 mg./Kg.; this compound in contrast to the others also showed some hypertensive effects.

EXPERIMENTAL[1]

2-(2-[N-Methyl-N-*i*-propylamino]ethoxy)-1-phenylethanol.—Sodium, 0.1 Gm. (0.005 gram atom) was added to 46.8 Gm. (0.4 mole) of N-methyl-N-isopropylaminoethanol while stirring. After solution was complete, the temperature was raised to 80° and 24.0 Gm. (0.2 mole) of styrene oxide added over one and one-half hours. Stirring was continued at this temperature for an additional five hours.

On distillation, 23.5 Gm. of N-methyl-N-isopropylaminoethanol was collected, b. p. 72° (30 mm.), followed by 28 Gm. (60%) of product, b. p. 120–128° (0.35 mm.).

[1] Typical examples of the procedures used are given. Data shown in the tables are not reproduced. Initial reactants were all obtained from commercial sources.

TABLE I.—DIALKYLAMINOALKOXYPHENYLETHANOLS

$R_1R_2N\text{-}Y\text{-}O\text{-}CH_2CH(R_3C_6H_4)OH$[a, b]

Compd. No.	R_1	R_2	B. P., °C. (mm.)	Formula	Analyses[c] Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
1	CH_3—	*i*-C_3H_7—	120–128 (0.35)	$C_{14}H_{23}NO_2$	70.9	71.1	9.8	9.4	5.9	5.6
2	HCl[d]			$C_{14}H_{24}ClNO_2$	61.4	61.1	8.8	8.3	5.1	5.4
3	C_2H_5—	C_2H_5—	131–134 (0.6)	$C_{14}H_{23}NO_2$	70.9	70.8	9.8	10.2	5.9	5.8
4[bα]	C_2H_5—	C_2H_5—	118–120 (0.1)	$C_{15}H_{25}NO_2$	71.7	71.7	10.0	9.7	...	...
5	—$(CH_2)_4$—		130–138 (0.1)	$C_{14}H_{21}NO_2$	...	...	...	...	6.0	5.7
6	HCl[e]		...	$C_{14}H_{22}ClNO_2$	61.9	62.1	8.2	8.3	5.2	5.5
7	—$(CH_2)_5$—		148–158 (0.4)	$C_{16}H_{25}NO_2$	73.0	73.2	9.6	9.6	...	...
8	*n*-C_4H_9—	*n*-C_4H_9—	136–145 (0.05)	$C_{18}H_{31}NO_2$	73.7	73.7	10.7	10.7	4.8	4.8
9[aα]	CH_3—	CH_2—	122–127 (0.1)	$C_{13}H_{21}NO_2$	69.9	70.1	9.5	9.2	...	...
10[aβ]	C_2H_5—	C_2H_5	98–102 (0.04)	$C_{15}H_{25}NO_2$	71.7	71.8	10.0	9.8	5.6	5.5

[a] Y = —$(CH_2)_2$— unless otherwise shown; [aα] —$CHCH_3CH_2$—; [aβ] —$(CH_2)_3$—. [b] R_3—C_6H_4— = phenyl unless otherwise shown; [bα] *p*-tolyl. [c] Analyses are by Weiler and Strauss, Oxford, England. [d] Hydrochloride of compound immediately above, m. p. 133–137° (methyl ethyl ketone). [e] *Ibid.*, m. p. 125–130° (methyl ethyl ketone).

TABLE II.—ESTERS

(See Formula I), Y = —$(CH_2)_2$—[a, b]

Compd. No.	R_4	B. P., °C. (mm.)	Formula	Analyses Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
1	H	162–164 (0.06)	$C_{21}H_{27}NO_2$	73.9	74.1	8.0	8.3	...	...
2[bα]	H	176–180 (0.2)	$C_{22}H_{29}NO_2$	74.3	74.1	8.2	8.1	3.9	3.6
3	*p*-CH_2^-	156 (0.03)	$C_{22}H_{29}NO_2$	74.3	73.8	8.2	8.2	3.9	3.7
4	*o*-CH_3O^-	178–180 (0.08)	$C_{22}H_{29}NO_4$	71.1	71.8	7.9	8.2	3.8	3.2
5	*p*-CH_3O–	188–192 (0.2)	$C_{22}H_{29}NO_4$	71.1	71.2	7.9	7.8	3.8	3.8
6	*p*-Cl–	198–200 (0.5)	$C_{21}H_{26}ClNO_2$	67.1	67.3	7.0	7.2	3.7	4.0
7	[c]	192–194 (0.08)	$C_{29}H_{29}NO_2$	75.2	74.9	8.0	7.9	3.8	3.8
8[aα]	H	160–162 (0.04)	$C_{21}H_{27}NO_2$	...	...	...	...	4.1	3.9
9[aβ]	H	146–150 (0.05)	$C_{21}H_{25}NO_2$	...	...	...	...	4.1	4.2
10[aγ]	H	187–190[d]	$C_{21}H_{26}ClNO_2$	...	...	...	...	3.5	3.4
11[aδ]	H	161–164 (0.005)	$C_{25}H_{25}NO_2$	75.5	75.9	8.9	9.1	3.5	3.5

[a] R_1, R_2 = C_2H_5– unless otherwise shown; [aα] R_1 = CH_3–, R_2 = *i*-C_3H_7–; [aβ] R_1 + R_2 = —$(CH_2)_4$—; [aγ] R_1 + R_2 = —$(CH_2)_5$—; [aδ] R_1R_2 = *n*-C_4H_9–. [b] R_3 = hydrogen unless otherwise shown; [bα] *p*-CH_3–. [c] Aryl group is cinnamoyl. [d] Melting point (isopropyl alcohol) of hydrochloride.

TABLE III.—ANESTHETIC EFFECTS[a]

Compd. No.	ED₅₀, mg./ml.	$LD_{min.}$, mg./Kg.	Reference Series[b] ED₅₀, mg./ml.	Reference Series[b] $LD_{min.}$, mg./Kg.
1	0.2	1000	0.32	>1,000
2	0.66	750	6	1,000
3	0.66	250	1.3	>1,000
4	0.4	200	0.17	400
5	0.06	150	[c]	750
6	0.26	750	1.3	>1,000
7	0.28	750	0.6	400
8	0.68	300	0.7	1,000
9	0.08	750	0.45	1,000
10	4.3	1000	0.88	1,000
11	2.6	750	0	>1,000
C[d]	13	200	...	...

[a] The anesthetic effect (ED_{50}) and toxicity ($LD_{min.}$) were established as described in (4). [b] These compounds have all R substituents the same as compounds in this series, and have been described (4); they differ from the present series in lacking the -O-Y- structural feature of the general formula I. [c] Not evaluated. [d] C = procaine introduced as a control.

2-(2-Diethylaminoethoxy)-1-phenylethyl Benzoate.—To a stirred refluxing solution of 4.2 Gm. (0.03 mole) of benzoyl chloride in 100 ml. of benzene was added 7.1 Gm. (0.03 mole) of 2-(2-diethylaminoethoxy)-1-phenylethanol over fifteen minutes. Reflux was continued for two hours and the solvent removed. The oily residue was taken up in 100 ml. of water, basified with 40% aqueous sodium hydroxide, and extracted with five 20-ml. portions of ether. The combined ether extracts were dried (anhydrous magnesium sulfate), filtered, and the solvent evaporated. Distillation of the residue gave 4.8 Gm. (47%) of product, b. p. 162–164° (0.06 mm.).

Isolation of Acid-Insoluble Products (Compound 10, Table I.)—In the preparation of compound 10, Table I, the fractions boiling at 118–138° (0.04 mm.) were dissolved in ether and the amino alcohol extracted with 3 *N* hydrochloric acid. The ether layer, containing the acid-insoluble fraction, was dried (anhydrous magnesium sulfate) and distilled. Styrene glycol (confirmed by mixed m. p. with authentic material) was collected at 132–142° (3 mm.) and a second product (fraction II) at 120–130° (1 mm.). In several runs, a high-boiling fraction III was obtained, b. p. 158–170° (0.05 mm.).

Fraction II, on redistillation, boiled at 106–108° (0.02 mm.). $\lambda_{242}^{e1\%}$ (95% ethanol) 169. *Anal.*—Found: C, 76.6; H, 7.1.

Fraction III, on redistillation, boiled at 144–150° (0.01 mm.) $\lambda_{262}^{e1\%}$, 257, 252 (95% ethanol) 34.8, 33.1, 28.5. *Anal.*—Found: C, 70.1; H, 7.4.

Fractions II and III slowly decolorized a solution of bromine in carbon tetrachloride with no evolution of hydrogen bromide, and have not as yet been characterized.

2-(2-Diethylaminoethoxy)acetophenone.—Sodium, 6.9 Gm. (0.3 gram atom), was dissolved in a solution of 35.1 Gm. (0.3 mole) of 2-diethylaminoethanol in 350 ml. of toluene. The solution, stirred and maintained at reflux, was treated with a solution of 19.9 Gm. (0.1 mole) of 2-bromoacetophenone in 100

ml. of toluene over a period of four hours. After an additional eight hours under reflux and cooling, the reaction mixture was diluted with 20 ml. of ethanol and 250 ml. of water. The aqueous layer was extracted with two 100-ml. portions of toluene followed by two 100-ml. portions of ether, the organic phase combined with the initial toluene layer, the whole washed with 0.5 L. of 0.6 *N* hydrochloric acid, the washings basified (pH 10), and the formed oil extracted with ether. After drying (anhydrous magnesium sulfate) and removal of solvent, distillation gave 1.8 Gm. (8%) of product, b. p. 116–120° (0.05 mm.), (n_D^{25} = 1.5136).

Anal.—Calcd. for $C_{14}H_{21}NO_2$: C, 71.5; H, 9.0; N, 6.0. Found: C, 71.1; H, 9.6; N, 6.5.

2-(2-Diethylaminoethoxy)-1-phenylethanol (14).—A solution of 3.3 Gm. (0.014 mole) of 2-(2-diethylaminoethoxy)acetophenone in 250 ml. of ethanol was quantitatively (one equivalent) hydrogenated over three 6-hour shaking periods with initial pressure of four atmospheres, using three 0.2-Gm. portions of 10% palladium-on-carbon as catalyst, and heating at a jacket temperature of 50°. After filtration and removal of solvent, there was obtained 2.0 Gm. (61%) of product, b. p. 119–122° (0.25 mm.), (n_D^{25} = 1.5090).

Anal.—Calcd. for $C_{14}H_{23}NO_2$: C, 70.9; H, 9.8; N, 5.9. Found: C, 70.5; H, 9.8; N, 5.9.

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Relative Potencies of Some Phenothiazines as Pecking Syndrome Inhibitors

By A. M. BURKMAN

Members of a select group of chlorinated, fluorinated, and unhalogenated phenothiazines of known (i.e., reported) antiemetic potency in dogs were evaluated as pecking syndrome antagonists in pigeons. Assigning to chlorpromazine an arbitrary potency value of 1.0, it was calculated that promazine = 0.1, prochlorperazine = 2.3, triflupromazine = 4.4, trifluoperazine = 7.2, and perphenazine = 10.2. The relationship of these data to the reported antiemetic activity of the compounds is discussed.

THE phenothiazines possess many diverse and apparently independent actions which require equally diverse methods for their quantitation. Of particular interest to us are those laboratory procedures designed to evaluate antiemetic activity since antiemesis represents one of the more important therapeutically useful characteristics of the phenothiazines.

The most frequently employed laboratory method for both screening and quantitatively evaluating antiemetics involves the administration of predetermined ED_{99} or "threshold" doses of apomorphine to dogs premedicated with the drug being investigated (e. g., 1–3). The use of the dog has been as much a matter of necessity as choice inasmuch as most of the other familiar laboratory animals are quite refractory to the emetic effects of apomorphine. The pigeon is one such animal. This bird has been the subject of our special attention because of its unusual, albeit nonemetic, response to apomorphine. The behaviorally distinct pecking syndrome in pigeons, described elsewhere (4–7), has been shown to be markedly influenced by several antiemetic substances, most notably the chlorinated phenothiazines (8). It has been observed that the more effective antiemetic agent (apomorphine antagonist in dogs) appeared to be the more efficient pecking syndrome inhibitor (apomorphine antagonist in pigeons).

The present communication deals with quantitation of antipecking activity of a select group of

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TABLE I.—RELATIVE POTENCIES OF PHENOTHIAZINES

Drug	ID_{50}[a] (95% C. L.)[b]	Slope Function[c] (95% C. L.)	Potency Ratio (95% C. L.)
Promazine	205 (137–308)	2.4 (1.3–4.4)	0.1 (0.05–0.18)
Chlorpromazine	20.5 (12.1–32.8)	2.4 (1.3–4.1)	1.0
Prochlorperazine	8.50 (6.00–12.0)	2.3 (1.5–3.5)	2.3 (1.3–4.1)
Triflupromazine	4.70 (3.10–9.10)	2.4 (1.3–4.3)	4.4 (2.4–8.0)
Trifluoperazine	2.85 (1.80–4.56)	2.4 (1.4–4.2)	7.2 (3.7–14)
Perphenazine	2.00 (1.20–3.30)	2.5 (1.3–2.7)	10.2 (5.2–20)

[a] Quantity of drug (10^{-7} moles/Kg.) necessary to produce a 50% reduction in control pecking syndrome elicited by 0.5 mg./Kg. of apomorphine hydrochloride, i. p. [b] Confidence limits ($p < 0.05$). [c] Antilog $(X_2 - X_1)/(Y_2 - Y_1)$.

TABLE II.—RELATIVE POTENCIES OF PHENOTHIAZINES AS ANTIEMETICS IN DOGS

Drug	Route	Challenging Dose of Apomorphine HCl, mcg./Kg.	Relative Potency[a]	Reference
Promazine	i. v.	22.7, i. v.	0	(10)
Prochlorperazine	i. v.	22.7, i. v.	3	(10)
Prochlorperazine	s. c.	100, s. c.	6	(11)
Triflupromazine	s. c.	52–167, i. v.	10	(12)
Trifluoperazine	p. o.	10, i. v.	18	(13)
Perphenazine	i. v.	22.7, i. v.	24.2	(10)
Perphenazine	i. v.	100, i. v.	16.5	(14)
Perphenazine	i. v.	500, i. v.	47.4	(14)

[a] Chlorpromazine = 1.0.

fluorinated, chlorinated, and unhalogenated phenothiazines, several of which are well established antiemetics.

METHOD

Adult domestic pigeons (450–700 Gm.) of both sexes were initially screened for apomorphine sensitivity at the 0.5 mg./Kg. level (i.p.). Previous work has established that at this dose approximately 90% (a theoretical ED_{98}) of an arbitrary sampling of birds display a vigorous pecking syndrome without ill effect (5). The nonreactors were rejected as unsuitable for the assay. Cumulative (total) pecking responses to this dose of apomorphine were recorded and quantitated and these served as control values.

Groups of 12 birds were premedicated with geometrically spaced doses of phenothiazines and pecking syndrome characteristics monitored during the following two hours. The mean cumulative responses so recorded[1] were compared with mean control values for the same group of birds. Thus each pigeon essentially served as its own control; and the effect of each drug expressed as per cent inhibition of the control response. Doses of the phenothiazines were calculated as moles of base per kilogram of body weight, and all were administered by the intraperitoneal route. The challenging dose of apomorphine hydrochloride remained a constant (0.5 mg./Kg.) throughout the experiment.

Fifteen minutes elapsed from the time of premedication to the time of apomorphine challenge.

Every point used to plot the dose-response lines represents a mean value for 12 birds. The median inhibitory dose (ID_{50}) for each of the drugs was read directly from the graph and relative potencies calculated (assigning to chlorpromazine an arbitrary value of 1.0) according to Litchfield and Wilcoxon (9).

[1] One-minute pecking counts were taken at five to ten-minute intervals and plotted against time. The area under such a curve (measured with the aid of a polar planimeter) represents the cumulative response for a single bird. Since the pecking syndrome is easily interrupted by sudden noises that distract the bird, it becomes necessary to conduct the experiment in a sound-proof environment.

RESULTS AND DISCUSSION

The dose-response lines for each of the six premedicants are presented in Fig. 1. The lines, which do not significantly vary from parallelism (see slope function, Table I), were tested for "goodness of fit" and found to be acceptable. The corresponding median inhibitory doses (ID_{50}'s), which were derived from this graph, and their confidence limits are listed in Table I along with their potency ratios. The drugs are ranked in order of increasing potency.

Within the range of dosages employed, only promazine gave evidence of toxicity. At approximately 200 × 10^{-7} moles per Kg. (and higher), promazine induced increasingly severe retching, vomiting, ataxia, and excitement. These symptoms interfered with, overwhelmed, and eventually com-

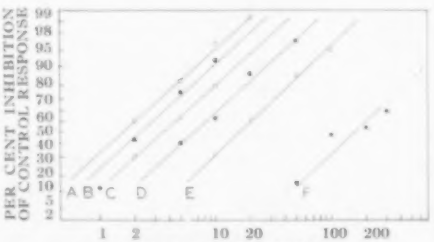

Fig. 1.—Dose-response lines for six phenothiazines: *A*, perphenazine; *B*, trifluoperazine; *C*, triflupromazine; *D*, prochlorperazine; *E*, chlorpromazine; *F*, promazine. Symptoms of toxicity prevented the measurement of antipecking activity of promazine at high dose levels hence the interrupted portion of the dose-response line is a theoretical extrapolation.

pletely obliterated the pecking syndrome evoked by apomorphine so that beyond this dose level the pecking response could not be measured. The upper portion of the dose-response line of promazine is, therefore, a theoretical extrapolation and is so indicated by a series of dashes (Fig. 1 *F*). All other lines followed a characteristic sigmoid pattern when plotted in arithmetic dimensions; a linear pattern when plotted logarithmically.

The order of potency of these six phenothiazines as pecking syndrome antagonists appears to agree with their reported effectiveness as antiemetics in dogs; the order being perphenazine > trifluoperazine > triflupromazine > prochlorperazine > chlorpromazine > promazine (Table II). The relative potencies of these drugs as pecking syndrome inhibitors, however, exhibit a somewhat more conservative range than they do as antiemetics. Incontrovertible parallelism of antipecking and antiemetic activity, however, cannot be proved at this time inasmuch as data from standardized antiemetic protocols are not as yet available. In Table II is summarized the type of information to be found in the literature: relative antiemetic potencies of various phenothiazines. Each was compared against chlorpromazine, but the antiemetic end point used by each investigator is not always identical; nor are the doses of the challenging emetic the same. This severely limits the applicability of these data to comparative studies such as this.

For instance, the relative potencies are known to vary with the dose of apomorphine used as the challenging emetic in dogs. Thus, in Table II, we have more than one estimate of the activity of perphenazine. Its potency, determined by Wang (14) in dogs, does indeed vary depending upon the magnitude of the challenge. Unless a "threshold," or ED_{99}, or some equally standardized dose is employed the potencies determined by different investigators are not readily comparable.

Similarly, one may suspect that the use of a dose of apomorphine other than 0.5 mg./Kg. used in this study might yield a different array of relative potencies. Attempts to confirm this suspicion are now in progress. However, the choice in this instance was not an entirely arbitrary one. Previous studies (5) on the dose-response relationship of apomorphine in pigeons assured us of a high incidence (ED_{99}), maximal pecking rate (110/min.), convenient duration (fifty-six minutes), and lack of side effects. These factors contributed to its selection.

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Incremental Thermal Titrations

By F. M. GOYAN, R. D. JOHNSON, and R. H. BLACKWOOD

An automatic pipet is used with external glass check valves to deliver equal increments of a titrating agent from one Dewar flask to another one containing the dissolved sample. Each increment of titrant causes a slight change in temperature due to the heat of the reaction occurring. By using a recording potentiometer to establish equal time intervals and to plot the resulting temperature changes, end points are easily identified as the last increment causing a characteristic recording. An alternative method can be used with the same equipment. This is a differential approach which allows the recorder to respond to any change in temperature, but to return to some base line before the regular time for the next increment. The usefulness of both methods is illustrated by a titration of 0.02 *M* boric acid with and without glycerol.

THERMAL TITRATIONS are performed by observing small changes in temperature accompanying the regular addition of various titrating agents. When the reaction involved in the titration is completed, the corresponding heat of reaction no longer contributes to the change in temperature, thus producing a marked break in the curve derived by plotting some function of temperature against the quantity of titrant added. Reactions that go to completion and give off or absorb heat adequately can be followed without the need for specific

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indicators or electrodes. The curves obtained can be used to determine heats of reaction and of solution (1, 2).

Linde, Rogers, and Hume (3) published a paper in which they surveyed early work and significantly advanced the method by the use of a thermistor bridge and recording potentiometer in combination with a constant flow device for adding titrant. Previously, a committee on nomenclature of the American Chemical Society (4) specifically recommended against the use of the term "thermometric" in this connection without suggesting acceptable nomenclature. In a recent review, Jordan (5) used the term "thermochemical" but suggested that the term "thermometric" was again in favor and that it might later give place to the use of the word "enthalpy" as an adjective describing these titrations. The word "thermal" has also been used in the same way (6).

In spite of the confusion in nomenclature, the usefulness and validity of these titrations has been established for several general reactions, including the neutralization of weak monoprotic acids (7), chelation with EDTA (2), and the reaction of water with acetic anhydride in acetic acid (8). There can be no doubt that the method can be used for many routine assays and that it lends itself to the quantitative study of reactions for which suitable electrodes or indicators are difficult to find.

The authors began exploring this method with the idea of adapting it to pharmaceutical assay using existing equipment. The new method which evolved is based upon the automatic addition of predetermined equal volumes of titrant at equal time intervals. Each increment of titrant produces a temperature change which shows distinctly on the chart of a recording potentiometer. The record between additions indicates normal temperature drifts.

Another advantage of the incremental additions of titrant is the simplicity of the arrangement necessary for differential operation. A reference thermistor or thermocouple junction in the titration vessel is sufficiently well insulated thermally that any change in temperature is recorded immediately by the normal element in thermal contact with the solution. However, the insulation is adjusted so that the reference element has time to approach the temperature of the solution during the interval between increments. The resulting record consists of a series of pronounced pips which terminate abruptly at the end point.

The automatic recording of temperature differences rather than a cumulative record of temperature change permits the use of higher sensitivity without danger of exceeding the limits of the recorder chart. However, the cumulative method is easier to relate to absolute heat effects and to the estimation of fractional response at the end point. Both the differential and the cumulative method are based upon the incremental addition of titrant.

EXPERIMENTAL

Materials.—All chemicals used were of reagent quality with the exception of the sodium hydroxide which was further purified by dissolving it in an equal weight of water and removing the precipitated sodium carbonate by filtration. Solutions made by the dilution of this 50% mixture were titrated using potassium acid phthalate as the primary standard. The boric acid solution used was diluted from the stock 0.2 *M* boric acid and 0.2 *M* potassium chloride used in preparing buffer standards.

Temperature Recording.—The recording potentiometer and amplifier system used in this work has been described (9). For this work, the chart speed was set approximately to 1 cm./min. and the filter network between amplifier and recorder was changed as shown in Fig. 1 by incorporating a 250 mfd. electrolytic capacitor in series with a single penlight battery. Need for a second capacitor is eliminated by negative feedback through a 10 megohm resistor between the input and output of the amplifier. Various modifications of the circuit shown in Fig. 1 have been used successfully during the course of the work.

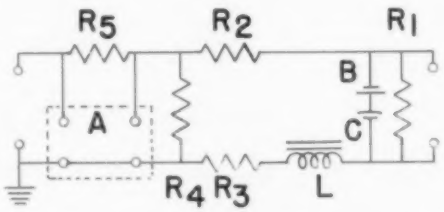

Fig. 1.—Filter system between amplifier and recorder. *A*, Amplifier; *B*, penlight battery (1½ v.); *C*, electrolytic capacitor (250 mfd.); *L*, choke (320 hy.); R_1–R_5, resistors, 500 ohms, 10,000 ohms, 47 ohms, 700 ohms, and 10 megohms, respectively.

A copper-constantan thermocouple and a thermistor bridge were used at different times for sensing temperature. An attempt to maintain the reference junction of the thermocouple in a matching Dewar flask during the titrations proved to be needlessly complex although effective. During this phase of the work, the incremental feature was not used to its fullest extent nor was its unique value appreciated. The thermistor bridge was introduced later when it seemed desirable to be able to switch at will from the cumulative to the differential method of recording.

The bridge was a conventional Wheatstone bridge, two arms of which were the 30-ohm sections

of a General Radio ratio arm box. The other two arms were thermistors having resistance values of approximately 2,000 ohms at room temperature (No. 32-A-1, Victory Engineering Corp.). The thermistor with the lowest resistance was chosen as the reference element and was connected in series with a variable resistor and decade resistance box for bridge balancing. The three elements, reference thermistor, resistor, and box, were replaced by a second decade resistance box for cumulative temperature recording. Both thermistors were held in place in a Pyrex tube (18 cm. × 9 mm. o. d.) which tapered to a thin-walled bulb (4 mm. o. d.) containing a drop of mercury. The thermistor used for normal measurement was held in the mercury in thermal contact with the solution by means of a packing of glass wool, which also served to keep the mercury in place and to insulate the reference thermistor and hold it about 2–3 cm. above the tip.

The amplifier was connected across the two resistors of the ratio arm box. The bridge was powered with a $1^1/_2$ v. No. 6 dry cell and was built around a heavy-duty Leeds and Northrup double-pole, double-throw switch, one side of which was used to select the mode of operation (cumulative or differential) and the other side was arranged so that the neutral position disconnected the battery. The design of this circuit was suggested entirely by availability of components, including the amplifier, which is not an absolute requirement when working with thermistors. However, the amplifier proved to be convenient for selecting full-scale sensitivity ranges from 0.05 to 0.2°. The higher sensitivities are especially useful for work with very dilute solutions or when using the differential method to display maximum effects.

The bridge was balanced in the usual way after adjusting the amplifier with the switch on the bridge at the neutral position The amplifier could be used as a nul meter, reading resistance directly from the second decade box. Initial balance for the differential method was obtained in the same way except that the box and variable resistor in series with the reference thermistor was used. After initial balance, changes in temperature were followed on the chart of the recording potentiometer without further adjustment of the resistance boxes except to prevent exceeding the scale limits of the recording potentiometer.

Incremental Additions.—A Brewer automatic pipetting machine (model 40) was equipped with a microswitch operated by an extrusion attach.d to the inner rotating drum by means of a metal band which allowed for the adjustment of the position of the extrusion. Suitable adjustment of this band caused the microswitch to be activated at the time of the completion of the cycle of the syringe. The switch was placed in the circuit in a normally closed position so that contact opened a latching circuit of a relay which was brought into the latched position upon the completion of a contact in the recording potentiometer. (A Leeds and Northrup commutator assembly supplied this contact at regular intervals.)

Contacts on the relay were connected across the main switch of the pipetting machine, causing it to complete one full cycle after each contact from the commutator assembly which itself was of shorter duration than the machine cycle. Details of powering the relay by means of a selenium rectifier built inside the case of the pipetting machine are immaterial.

The valves were removed from the pipetting machine. The syringe and other space for fluid were filled with mercury. Thus modified, the pipetting machine merely produces alternating surges of mercury which are transmitted to the titrant. In most of this work a 1-ml. syringe was set to deliver about 0.1 ml. every forty seconds.

Figure 2 shows the system used to convert surges provided by the pipetting machine (*P*) to repeated incremental additions of titrant from the delivery tip (*T*). The connection between the mercury compartment (*M*) and the check-valve assembly is made with one-eighth-inch black rubber tubing of sufficient length and flexibility that the flask containing the titrating agent can be moved about freely. When the fluid is moving toward the valve assembly, the upper valve opens a direct path to the delivery tip. Movement of fluid in the reverse direction closes this valve and opens the lower valve. A narrow-mouth Dewar flask (not shown) contains the titrant and the entire valve assembly which is held in place with a rubber stopper. The valve assembly is designed to contain a large volume compared to the connecting tubes, with the result that only fluid which is stored in the flask is transported to the delivery tip. A study of colored fluids shows negligible transfer between the Dewar flask and the mercury

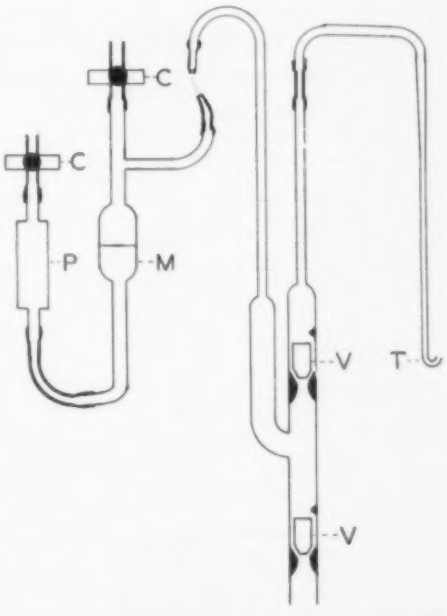

Fig. 2.—A schematic diagram of the check-valve assembly. *P*, Valve housing of pipetting machine with valves removed; *M*, mercury chamber where titrant contacts mercury; *C*, conventional screw clamps; *V*, ground-glass check valves; *T*, delivery tip.

reservoir. The crook on the tip is another important detail of design. It prevents dense titrants from being displaced by gravity alone.

Details concerning the most effective construction of check valves are not final. In the present assembly, the valves (*V*) were shaped to a carbon rod sharpened in a pencil sharpener. They were ground to fit exactly, using a graded series of abrasives. The grinding was done with the help of a stirring motor before the valves were cut from the Pyrex rod from which they were made. Other attempts to make valves with different angles failed. Commercially available Pyrex balls are scheduled for future trial.

Operating Procedure.—The titrating agent selected for use is poured into a 400-ml. narrow-mouth Dewar flask into which the check valve assembly shown in Fig. 2 is secured by means of a rubber stopper fitted with a soda-lime tube if needed. Usual analytical precautions as to rinsing, etc. are observed. The valve assembly is filled by suction applied by mouth to a pipet fitted temporarily into the rubber tubing above the mercury reservoir (*M*). When it is observed that all air has been removed, the screw clamp is closed and the pipet removed and set aside. The pipetting machine is then switched on and allowed to operate at a reasonably fast rate until the air in the outlet compartment has been driven out through the delivery tip. When all of the air has been removed by suction and by rapid delivery, the main switch is turned off and the connection to the commutator in the recorder allowed to initiate complete cycles at regular intervals. The instrument is set for a slow cycling speed and increments are delivered into a waste jar until the tip is placed in the titration vessel at the start of a titration.

A 200-ml. narrow-mouth Dewar flask is selected as a titration vessel and clamped in such a way that the tube containing the temperature-sensing elements and a screw-type glass stirrer is centrally and permanently located near the bottom of the flask by means of conventional clamps. One ring stand is used to support the stirring motor, the thermistor assembly, and the titration vessel. The clamp holding the titration vessel can be loosened so the vessel can be removed for filling and replaced in the same position. The stirring motor operates up to 1,600 r. p. m. and it was found that the rate of stirring is not critical but that fast stirring is desirable with the small glass screw made to fit the assembly.

The sample selected for titration is transferred to the flask and sufficient water added to bring the total volume to about 100 ml. The flask is clamped in place and the stirrer and temperature recorder started. As soon as the bridge is balanced and other necessary adjustments made to secure a reasonably steady base line on the recorder, the delivery tip is wiped with a piece of filter paper and inserted into the titration vessel under the surface of the solution being titrated. This operation is easily accomplished between automatic deliveries of titrant.

RESULTS

This paper is intended to present the incremental method of thermal or thermometric titration.

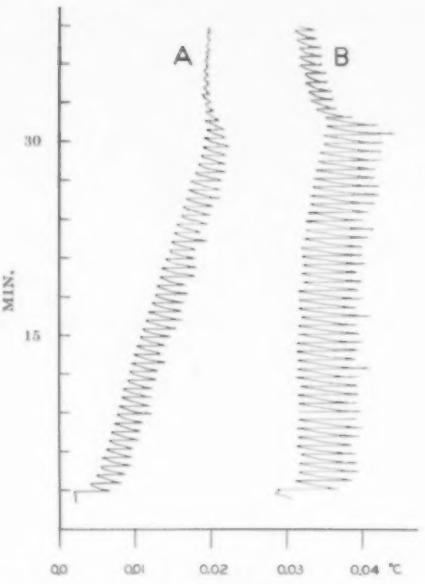

Fig. 3.—A tracing of chart records obtained in titrating 10 ml. of 0.2 *M* boric acid with 0.5 *M* sodium hydroxide by the differential method. *A*, Boric acid diluted with 90 ml. of pure water; *B*, boric acid diluted wih 90 ml. of 50% glycerol.

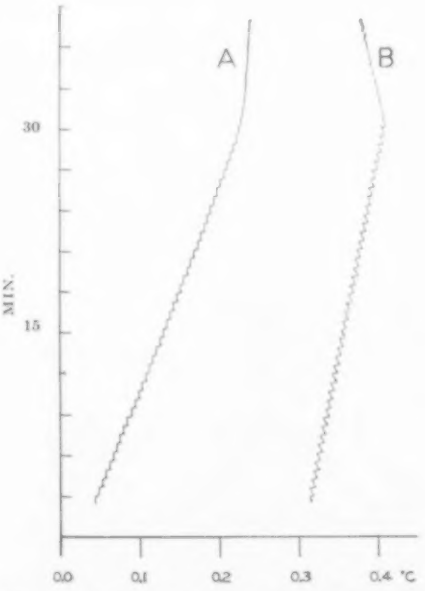

Fig. 4.—A tracing of chart records obtained in titrating 10 ml. of 0.2 *M* boric acid with 0.5 *M* sodium hydroxide by the cumulative method using low temperature sensitivity. *A*, Boric acid diluted with 90 ml. of pure water; *B*, boric acid diluted with 90 ml. of 50% glycerol.

The authors do not imply by the choice of a title any recommendation as to nomenclature. This modification of the general method, regardless of nomenclature, offers the same basic limitations and advantages. However, the use of the check-valve assembly with a pipetting machine or other device to provide surges initiated by a switch in the recorder has interesting advantages, especially as it facilitates the differential method, the results of which are shown in Fig. 3. It will be noted that the temperature sensitivity used was ten times greater than that used for the cumulative method shown in Fig. 4. Temperature sensitivity may be selected by the operator for both methods but the differential method requires much less attention at high sensitivity.

The results shown in Figs. 3 and 4 represent the titration of 100 ml. of 0.02 *M* boric acid with 0.5 *M* sodium hydroxide. Each scale division on the ordinate axis represents about three minutes. Curve A on both graphs shows the results obtained without adding glycerol. This example was chosen to illustrate the power of the method over conventional pH titrations. It will be noted that the slight ambiguity at the end point in the titration of boric acid without glycerol can be minimized by using the curve well past the end point to establish normal drift. Much of this drift is from unknown causes but the major differences arise from the fact that no attempt was made to adjust the temperature of the solution being titrated, except by having it approximately at room temperature. One of the advantages of these methods is that such adjustment is not necessary.

Crude end point corrections may be based on the principle that after a "zero" correction for drift, incomplete temperature increments represent proportionally incomplete reaction due either to approach to equilibrium or to the exact end point requiring a fractional increment of titrant. With such corrections, the present technique is accurate to about 1%. It will be noted that the number of increments may be counted on the chart. This fact suggests the possibility of using a counter instead of a recording potentiometer, but experiments of this type have not been tried as yet.

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Study of Inverse Isotope Dilution Analyses of Chlortetracycline

By C. E. BRECKINRIDGE, Jr.† and J. E. CHRISTIAN

Chlortetracycline was quaternized using carbon^{14}-labeled methyl iodide of known specific activity. The resulting radioactive chlortetracycline methiodide was diluted by the addition of a known amount of nonradioactive chlortetracycline methiodide. The specific activity of a purified portion of the mixture was then determined. From these data, the quantity of chlortetracycline originally present was calculated. The developed procedure was applied to the determination of microgram quantities of chlortetracycline in blood.

THE PURPOSE of this investigation was to develop and study new and better analytical procedures applicable to the determination of microquantities of chlortetracycline in biological materials.

The great sensitivity and selectivity of the labeled-derivative method of inverse isotope dilution analysis led to the choice of this procedure as the analytical technique. The most complete review concerning isotope dilution analysis to appear is that by Pinajian, Christian, and Wright (1). In a subsequent paper by Christian and Pinajian (2), the various modifications of isotope dilution analysis were discussed as were the procedure, limitations, and mathematical equations applicable for each variation.

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† Present address: Health Physics Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.
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EXPERIMENTAL

A satisfactory derivative was prepared by reacting chlortetracycline free base with methyl iodide, thus quaternizing the tertiary amine group as described by Boothe, *et al.* (3). An excellent method for the conversion of chlortetracycline hydrochloride to the free base has been presented by Stephens, *et al.* (4).

Carbon^{14}-labeled methyl iodide may be prepared according to the method of Murray and Williams (5) and it may be purchased from a number of suppliers of C^{14}-labeled reagents. The radioactive reagent used in this work was purchased in 1-mc.

quantities.[1] The contents of the shipment container were frozen in the lower portion of the container using dry ice-acetone mixture. The neck of the container was fractured and, after thawing, the contents were transferred to a glass-stoppered 2-ml. volumetric flask using 1 ml. of unlabeled methyl iodide in small portions to effect the transfer.

The specific activity of the reagent was determined by placing 25 μL. of the solution in a tared Wheaton vial of 20-ml. capacity, and weighing on a microbalance to determine the amount of methyl iodide present. Phosphor solution (15 ml.), consisting of toluene as the solvent, PPO (4 Gm. per L.) as the phosphor, and POPOP (0.1 Gm. per L.) as the wavelength shifter, was then added and the activity was determined in the Packard Tri-Carb liquid scintillation spectrometer.[2] Five samples were handled in this manner and the results averaged.

Analysis at the 10, 1, and 0.1-mg. Levels.—Initial determinations of chlortetracycline were made using 10-mg. quantities. Six weighing bottles were dried and tared, and weighed again after approximately 10 mg. of chlortetracycline[3] base had been added to each. Tetrahydrofuran (200 μL.) was added and each bottle was shaken to completely dissolve the sample. Twenty-five microliters of the carbon^{14}-labeled methyl iodide (the labeled reagent) was added, each bottle sealed tightly with the glass stopper, and the reaction allowed to proceed at room temperature for seven days. At the end of this period, each bottle was weighed and approximately 100 mg. of carrier (previously prepared and purified nonradioactive chlortetracycline methiodide) was added. Each bottle was again weighed to determine the exact amount of carrier added. In order to attain thorough mixing of the contents, ethyl alcohol (absolute) was added dropwise, applying heat gently after each addition. After complete mixing, the solution was filtered into a test tube and petroleum ether (b. p. 90–100°) was added in an amount equal to the volume of alcohol added. The precipitate which resulted was dissolved by gently heating. Each test tube was stoppered, placed in a hot water bath (90–100°) on a hot plate, the heat turned off, and the entire system allowed to return to room temperature slowly to permit crystallization. The crystallization process was repeated and the crystals, obtained by centrifugation and decantation, were dried in an oven at 90°. Previous studies had shown that no significant change in specific activity occurred after the second crystallization, indicating purity of the product.

Following the second crystallization, the crystals were placed in tared Wheaton vials and weighed Phosphor solution (15 ml.) was added to each and the radioactivity was determined in the liquid scintillation spectrometer.

Additional analyses at the 1-mg. level and at the 0.1-mg. level were carried out without alteration of the above described procedure. Table I summarizes the data and results obtained.

Analysis at the 10-mcg. Level.—Gravimetric procedures to determine final specific activities were replaced by spectrophotometric analysis at the 10-mcg. level permitting the addition of smaller amounts of carrier which increased the sensitivity of the analysis.

A dilution of chlortetracycline base was prepared in tetrahydrofuran and appropriate aliquots containing 10 mcg. of the base were pipetted into each of 15 glass-stoppered volumetric flasks of 2-ml. capacity which were used as reaction vessels. The quaternization reaction was carried out as described previously. A dilution of chlortetracycline methiodide (carrier) in ethanol was prepared and one ml. containing one mg. of the salt was added by pipet. Gentle heat was applied by a water bath (50–60°) to insure complete, intimate mixing. The resulting carrier-diluted derivative was precipitated by adding petroleum ether dropwise. The precipitate was collected by centrifugation, dissolved in ethanol (absolute), and crystallized slowly by adding petroleum ether as previously described. Crystallization was repeated to insure constant specific activity. The crystals were collected, dried, and dissolved in approximately 3 ml. of methyl alcohol. Exactly 3 ml. of this solution was pipetted into a quartz cell and the concentration determined in the spectrophotometer at 376 mμ, a point of maximum absorbance. This solution was then quantitatively transferred from the cell into a Wheaton vial, the phosphor solution added to give a volume of 15 ml., and the radioactivity was determined in the liquid scintillation spectrometer. Table I summarizes the results obtained.

Analysis at the 1-mcg. Level.—In performing determinations at the 1-mcg. level, paper chromatography replaced crystallization as the purification technique, permitting the addition of smaller quantities (0.1 mg.) of carrier. Instead of precipitating the derivative as was done at previous levels, the contents were evaporated slowly by placing the flask in a water bath at approximately 50°. When the volume was reduced to approximately 50 to 100 μL., heating was discontinued and the flask again stoppered.

Whatman No. 1 paper "for chromatography" was cut into pieces 30 × 40 cm. and a light pencil line was drawn 4 cm. from one end. Two samples of chlortetracycline methiodide in ethanol were added in 5-μL. portions to a known position on this line. The contents of the flask was then added to the line in 5-μL. portions, 2 cm. apart. The strip was dried, formed into a cylinder, and stapled so that the edges did not touch. The cylinder was placed in a pie plate containing *n*-butanol saturated with water. Enough solvent was used to give a depth of 1 cm. The system was then placed in a tightly closed chromatographic chamber and allowed to develop in an ascending manner. After the solvent front had risen approximately 20 cm., the cylinder was removed, opened flat, and hung in the hood to dry. The known spots were located using an ultraviolet light and all of the sample lying in the same plane was removed by cutting the square transversely, thus forming a strip which was approximately 3 cm. wide. The known spots were cut from this strip and the remaining portion was prepared for elution by cutting it to a taper at one end. The strip was suspended from a glass rod and methanol was allowed to drip slowly onto the paper from a buret located above it. The eluted sample was

[1] Tracerlab, Inc., 1601 Trapelo Rd., Waltham 54, Mass.
[2] Packard Instrument Co., LaGrange, Ill.
[3] Supplied in generous quantities as the hydrochloride by Lederle Division of American Cyanamid, Pearl River, N. Y. The Lederle product is Aureomycin hydrochloride.

TABLE I.—SUMMARY OF RESULTS OF INVERSE ISOTOPE DILUTION ANALYSES OF CHLORTETRACYCLINE

Chlortetracycline Assayed, mcg.	Chlortetracycline Methiodide Added, mg.	Initial Specific Activity[a] of Chlortetracycline Methiodide, Counts/min./mg.	Final Specific Activity of Chlortetracycline Methiodide, Counts/min./mg.	Weight (W)[b] of Chlortetracycline by Analysis, mcg.	Corrected Wt.[c] of Chlortetracycline, mcg.	Error,[d] %
10,000	105.7	2,130	233.7	10,100	...	1.00[h]
1,000	17.9	2,130	145.4	1,010	...	1.00[h]
100	9.6	2,130	30.8	108	...	8.0[h]
10.0	1.00	62,934	768.0[f]	9.5	...	5.0
1.00	0.100	41,220	481.9[g]	0.91	...	9.0
10.0[e]	0.100	95,835	9419.2[g]	8.4	11.2	12.0[i]
1.00[e]	0.100	95,835	925.8[g]	0.76	1.01	1.00[i]
0.100[e]	0.100	95,835	83.8[g]	0.068	0.09	10.0[i]

[a] Specific activities (SpA) were determined by a combination of gravimetric and liquid scintillation counting techniques unless otherwise noted.

[b] $$W = \frac{\text{weight of carrier} \times \text{final SpA}}{\text{initial SpA} - \text{final SpA}} \times \frac{\text{mol. wt. of chlortetracycline (478.90)}}{\text{mol. wt. of chlortetracycline methiodide (620.84)}}$$

[c] Corrected by multiplying by the average correction factor for these three analyses, 1.33.
[d] Based on an average of 15 analyses in each case unless otherwise noted.
[e] Sample added to whole blood.
[f] Determined by a combination of spectrophotometric and liquid scintillation counting techniques.
[g] Determined using paper chromatography, spectrophotometry, and liquid scintillation counting techniques.
[h] Based on an average of 6 analyses.
[i] Based on the corrected value.

collected in a test tube marked to contain approximately 3.2 ml. After collecting this quantity, exactly 3 ml. of the solution was pipetted into a quartz cell and the concentration was determined in the spectrophotometer. The solution was transferred quantitatively to a Wheaton vial using the phosphor solution (approximately 12 ml.) for rinsing and the total radioactivity determined. The results are presented in Table I.

Analysis of Chlortetracycline in Blood.—To determine the applicability of the method to the analysis of chlortetracycline in blood, 0.5 ml. of oxalated rat blood was pipetted into each of 45 test tubes 1 × 10 cm. Each sample was then handled as follows: water (1 ml.) was added to increase the volume, then 10 mcg. of chlortetracycline was added to 15 test tubes, 1 mcg. to 15 tubes, and 0.1 mcg. added to the remaining 15 tubes. Chlortetracycline was obtained by preparing a dilution in tetrahydrofuran and then taking appropriate aliquots by pipet. After mixing well, an additional 3 ml. of water was added. The protein was precipitated with tungstic acid using 0.5 ml. each of 10% sodium tungstate and 0.67 *N* sulfuric acid solution according to the procedure described by Simmons (6).

Following the addition of 10 drops of methyl alcohol to decrease foaming, the samples were centrifuged and the supernatant liquid decanted. The residue was washed and centrifuged and the supernatant liquid again decanted. This process was repeated again using tetrahydrofuran as the washing agent to obtain all of the chlortetracycline. The decantations were placed in a 1.5 × 15 cm. test tube which was placed in a beaker of water on a hot plate at 70–80°, and the liquid allowed to evaporate slowly.

The resulting residue was reconstituted by the addition of tetrahydrofuran, dropwise, to effect complete solution. Approximately 250 µL. was required. The sample was then quantitatively transferred to a 2-ml. glass-stoppered volumetric flask using tetrahydrofuran for rinsing. From this point, the analysis was carried out as described for the 1 mcg.-level determinations except descending chromatography was used in the purification step since this procedure was demonstrated to be faster, and more compact spots were obtained. Normal butanol, saturated with water, was used as the solvent system and the aqueous phase used to saturate the butanol was placed on the bottom of the chromatographic chamber. Table I summarizes the results of the analyses conducted on blood samples. It will be noted that the average weight by analysis of the 15 samples at each level (0.1, 1.0, and 10.0 mcg.) is low by a common factor approximating 1.33 as the average correction factor.

SUMMARY AND CONCLUSIONS

1. Chlortetracycline was assayed quantitatively in 10-mg., 1-mg., and 0.1-mg. quantities using the inverse isotope dilution technique in combination with gravimetric methods.

2. The above method was improved in sensitivity by the incorporation of chromatography and ultraviolet spectrophotometry for the purification step and for the determination of the final specific activity, respectively.

3. Using the improved method, chlortetracycline was determined in 10-mcg. quantities with an average error of 5.0 per cent and in 1-mcg. quantities with an average error of 9.0 per cent.

4. In blood, the antibiotic was determined in 10-mcg. quantities with an average error of 12 per cent; in 1-mcg. quantities with an average error of 1 per cent; and in 0.1-mcg. quantities with an average error of 10 per cent.

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Toxic Saponin from *Elvira biflora*

By MARILDA M. de OLIVEIRA and SYLVIA O. ANDRADE†

A saponin isolated from *Elvira biflora* exhibited toxicity in tests with laboratory animals. Pharmacological tests indicated spasmogenic and hypotensive activity, and contact with isolated strips of guinea pig ileum caused an increase in tonus. After hydrolysis, four sugars were identified by paper chromatography. Echinocystic acid was identified as the crystalline compound and by three derivates.

INVESTIGATIONS of the poisonous plants of the State of Sao Paulo (1) led us to study the toxic effects on laboratory animals of *Elvira biflora* (*Compositae*) and the isolation of its active principle. It was found that the toxicity of *Elvira biflora* varied during the plant growth; it is higher at the flowering time, from April to June.

In acute toxicity tests, the material was administered to guinea pigs by intraperitoneal injection, by stomach tube, and, in few instances, intravenously (2).

Studies on the nature of the active principle indicated that it is a triterpenoid saponin (3). Hydrolysis of the saponin with acid in alcohol solution gave a saponin and five sugars: galactose, arabinose, xylose, rhamnose, and one which has not been identified.

The crystalline sapogenin melted at 319–324°. Analysis indicated the sapogenin has the formula $C_{30}H_{48}O_4$, and that it contains a free carboxyl group, as shown by titration with standard alkali. The presence of two hydroxyl groups was indicated by the formation of a diacetate, m. p. 279–282°. The sapogenin was converted into the methyl ester, m. p. 210–212°, and the diacetate methyl ester, m. p. 196–198°. The infrared spectra did not show the four characteristic bands of steroidal sapogenins (4).

Crude preparations of the toxic principle of the plant showed that: (*a*) it is highly hemolytic; (*b*) it contracted isolated strips of guinea pig ileum suspended in the usual manner in Tyrode's solution; (*c*) when lethal doses were injected intravenously into cats under anesthesia (urethan), a fall in blood pressure and respiratory failure were observed.

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EXPERIMENTAL

Extraction.—Mature leaves, picked and frozen immediately with crushed dry ice according to Hopponen and Gisvold (5), were powdered in a Wiley laboratory mill and stored in a deep freezer. The frozen material was extracted several times with water at 70°, the extracts being filtered through gauze. The total volume of the filtrate, representing a 4% solution of leaves on a dry weight basis, was treated, per liter, with 150 ml. of ether and then with 150 ml. of *n*-butanol, saturated with water, to remove fat and green material. Acetic acid was added to the aqueous solution (to pH 5) and then fresh egg white was added as a colloidal defecant. The mixture was heated to 50°, the coagulum was removed, and the solution filtered, giving an orange extract. The liquid was heated to 50°, and was extracted three times with *n*-butanol, using 200 ml. for each liter of liquid. The clear *n*-butanol extract was concentrated under reduced pressure at 55° to a small volume when a light-colored amorphous solid was obtained. This precipitate was centrifugated and washed with ether, then vacuum dried to a powder. The substance was soluble in water, methanol, ethanol 80%, and *n*-butanol, and insoluble in benzene, chloroform, ethyl ether, acetone, and methylisobutyl ketone. Biological tests (intraperitoneal injection in guinea pig) were run with all extracts. In the *n*-butanol (neutral) extraction, a small amount of the active substance was lost. The final yield was 15 Gm., representing 0.49% of the dried plant.

Qualitative tests on the active material gave the following results: (*a*) water soluble with foaming and opalescence; (*b*) Molish test, positive; (*c*) Seliwanoff test, negative; (*d*) fish (*Lebistes reticulatus*) placed in 0.05% solution of the test preparation died within twenty minutes; (*e*) a water solution of the saponin boiled for ten minutes with cholesterol, then injected into a guinea pig, did not kill the animal, although a similar solution, after boiling without cholesterol, was lethal; (*f*) a water solution did not hemolyze blood cells of the guinea pig in phosphate buffer; (*g*) Keller-Kiliani test, a pink color at the interface; (*h*) Mayer's reagent, negative.

Purification.—Two procedures were used to purify the crude saponin: (*a*) Powdered cholesterol (500 mg.) was added to a water solution of 100 mg. of saponin, the mixture was boiled, and then filtered with a filter aid. The addition product was heated with a solvent mixture (10 ml. ethanol, 5 ml. water, 7 ml. benzene) under reflux for thirty minutes and filtered. The phase containing the saponin was separated and treated with activated charcoal. The mixture was filtered on a Büchner

funnel with a filter aid and the alcoholic solution obtained was concentrated until foam formed, the 10 ml. of *n*-butanol was added, and the mixture was concentrated to a small volume under reduced pressure at 55°. The white precipitate obtained was separated by centrifugation. (*b*) The crude saponin was dissolved in methanol and precipitated by the addition of two volumes of ether. After centrifugation it was washed with ether and dried. This material was dissolved in 95% alcohol, treated with activated charcoal, and filtered. The solution obtained was evaporated to dryness. Chromatographic analysis of the purified saponin showed no disappearance of hemolytic spots.

Paper Chromatography.—The chromatograph was run with the crude saponin, using the descending technique, with Whatman No. 1 paper and the solvent mixture *n*-butanol-acetic acid-water (4:1:5). The chromatograms were dried at room temperature and sprayed with: (*a*) guinea pig blood cells in isotonic buffer phosphate—one spot was revealed; (*b*) aniline phthalate—no detectable sugar spot was found; (*c*) reagent prepared by mixing four parts of 2% aqueous sodium periodate and one part of 1% potassium permanganate—five brown spots appeared (6); (*d*) bromocresol green 0.04% in ethanol—acid spots were detected, one corresponding to the hemolytic spot.

For the biological detection of saponin, 20 mg. of material was chromatographed on paper 16 cm. wide. After development of the chromatogram, it was marked with pencil transversely in segments of 1 cm. width, the segments were numbered, and then two borderline ribbons (1 cm. wide × 40 cm. long) were cut along the paper and sprayed with potassium permanganate and sodium periodate.

After determining the zones of the spots, horizontal strips of paper were cut and extracted with about 15 ml. of methanol. The alcoholic fractions were dried. Samples of all the fractions were used in the following experiments: (*a*) estimation of activity upon guinea pig gut—only one sample contracted the ileum, it was washed with Tyrode's solution and after forty minutes the sensibility became normal to histamine and saponin; (*b*) test of hemolysis—only one sample was highly hemolytic; (*c*) intraperitoneal injection into guinea pig—only one sample was toxic, the animal died within four hours; (*d*) intravenous injection in atropinized rats under anesthesia (urethan)—only one sample produced a blood pressure fall that was fatal.

Samples of crude and cholesterol-precipitated saponin purified by paper chromatography were hydrolyzed and the hydrolysates were analyzed by chromatography for sugar and sapogenin composition. They showed the same sugars as crude saponin and the same spot as precipitated sapogenin.

Sugar.—Crude saponin (100 mg.) was dissolved in 155 ml. of 50% ethanol to which 0.86 ml. of sulfuric acid was added, and the mixture was refluxed for two and one-half hours. After removal of the sapogenin with chloroform the solution was neutralized with barium carbonate and concentrated under vacuum at 40°. The material was then analyzed by paper chromatography, using the following solvent mixtures: *n*-butanol-water-acetic acid (4:5:1) and pyridine-ethyl acetate-water (2:2:1). The results are given in Tables I and II.

TABLE I.—CHROMATOGRAPHIC RESULTS WITH BUTANOL-WATER-ACETIC ACID (4:5:1)

Spot	R_f	Sugar	R_f
A	0.068	...	...
B	0.125	D-Galactose	0.123
C	0.195	D-Arabinose	0.197
D	0.211	D-Xylose	0.214
E	0.320	L-Rhamnose	0.320

TABLE II.—CHROMATOGRAPHIC RESULTS WITH PYRIDINE-ETHYL ACETATE-WATER (2:2:1)

Spot	R_f	Sugar	R_f
A_1	0.568	D-Galactose	0.557
B_1	0.611	D-Arabinose	0.605
C_1	0.662	D-Xylose	0.657
D_1	0.712	L-Rhamnose	0.713

The papers were sprayed with aniline phthalate, Tollens' reagent, and potassium permanganate in 2% sodium carbonate solution.

Time of hydrolysis was prolonged (nine hours) to see if spot A was a disaccharide and similar results were obtained. Charcoal chromatography (7, 8) was used to separate mono-, di-, and trisaccharides. The unidentified sugar A came out with the monosaccharides, as indicated by paper chromatography of the three eluted fractions.

Sapogenin.—Crude saponin (1 Gm.) was dissolved in 25 ml. 50% ethanol, 50 mg. of calcium chloride was added, the mixture was adjusted to pH 8 with 1 *N* sodium hydroxide, filtered, and acidified with 5 ml. hydrochloric acid and refluxed five hours. A pasty gel formed at the start and changed to a white precipitate. After filtration and washing with water it was dried *in vacuo;* yield 220 mg. The sapogenin was dissolved in ether and purified through the sodium salt. The slightly colored sapogenin was clarified with charcoal in isopropanol and was recrystallized from this solvent. The sapogenin is soluble in methanol, ethanol, isopropanol, acetone, ethyl ether, and chloroform; practically insoluble in benzene. It gave the following results with the indicated qualitative tests: (*a*) Salkowski—yellow, then deep red; (*b*) Liebermann-Storch-Morowsky—pink, blue, and finally green; (*c*) Liebermann—To 1 mg. of sapogenin dissolved in 0.5 ml. of acetic anhydride was added 0.5 ml. of concentrated sulfuric acid: a pink color immediately appeared in the lower phase, a fluorescent yellow at the interface, and a blue color in the upper layer; (*d*) Noller-Smith-Harris-Walker—yellow, pink, red-purple, violet, brown-red (after some time); (*e*) a pale yellow with tetranitromethane.

The sapogenin sublimes at 240°. Despite repeated recrystallization and sublimation, the melting range[1] was 319–324° (in vacuum sealed Pyrex tube after drying at 170° *in vacuo*). See Table III. $[\alpha]_D^{25}$ + 37.7 ±2° in 95% ethanol.

Anal.[2]—Calcd. for $C_{30}H_{48}O_4$: C, 76.2; H, 10.2; neut. equiv., 475.9 (titration with 1*N* NaOH). Found: C, 75.39; H, 10.10.

Paper chromatography was run on the crude

[1] Melting points were determined on the Kofler block.
[2] The microanalyses were carried out by Microanalytical Laboratory Oxford, England.

TABLE III.—COMPARISON OF ELVIRAGENIN AND DERIVATIVES WITH ECHINOCYSTIC ACID FROM OTHER SAPONINS

	Bergsteinsson and Noller (13) m. p., °C.	Tschesche and Forstmann (14) m. p., °C.	Sannié, *et al.* (15) m. p., °C.	Elviragenin m. p., °C.
Acid	305–310	305–310	291–293	319–324
Methyl ester	213–214	216–217	210–212	210–212
Methyl diacetate	200–201	200–201	198–200	196–198
Diacetate	272–275	...	242–249	279–282

sapogenin by ascending technique, using the following developers: ethyl ether-toluene-ethanol-water (30:10:1:9), ethyl ether-benzene (1:1), and ethyl acetate-ethanol-0.05 *N* ammonium hydroxide (80:17:13.6) (9). Only one spot was detectable when the chromatograms were sprayed with guinea pig blood cells in isotonic buffer phosphate [instead of saline as Heftmann and Hayden (10)] or bromocresol green. No spot was formed with Tollens' reagent.

Two analyses by column chromatography using 50 mg. and 25 mg. of purified sapogenin, respectively, in 8 Gm. of silica prepared according to Gordon, *et al.* (11), were made, using ethyl ether as solvent in the first and ethyl ether and chloroform (1:1) in the second. Normal curves were obtained. Chromatographed (80 mg.) on 4 Gm. of alumina (Merck), and using acetone, acetone plus 1% acetic acid, and acetone plus 2% acetic acid, the sapogenin came out when eluted with acetone and 1% acetic acid. After crystallization from isopropanol and drying, it showed the same wide melting range, which did not change when vacuum dried over phosphorus pentoxide and sublimed.

In the course of this investigation we received an authentic sample of echinocystic acid. It was impossible to separate this sample and our sapogenin by paper chromatography using the following solvents: ethyl acetate-ethanol-ammonium hydroxide (80:17:13.6); ethyl ether-toluene-ethanol-water (30:10:1:9); *n*-butanol-acetic acid-water (40:10:50); octanol-amyl alcohol-pyridine-formamide (6:2:1:4); *n*-butanol-ethanol-water (1:1:4) and (1:1:5) (12), and revealed with Liebermann-Burchard reagent (9).

Diacetate.—Acetylation was carried out with acetic anhydride and anhydrous sodium acetate by refluxing, pouring in water, extracting with ether, and evaporating to dryness. The residue was dissolved in methanol, decolorized with charcoal, refluxed with diluted methanol, and recrystallized six times from this solvent. The product gave a yellow color with tetranitromethane. Paper chromatography, using the same solvent used for sapogenin, developed only one spot.

Melting point 279–282°. $[\alpha]_D^{25} - 14.6°$ (chloroform). Samples were dried *in vacuo* over phosphorus pentoxide.

Anal.—Calcd. for $C_{29}H_{46}O_2(CH_3CO_2)_2CO_2H$: C, 73.3; H, 9.4. Found: C, 72.96; H, 9.41.

Methyl Ester.—Prepared with diazomethane in ether solution, recrystallized from methanol, and vacuum dried at 110°, the product melted at 240–260°. After six recrystallizations, it showed m. p. 210–212°. The same melting point was recorded after chromatography on alumina, and it was not depressed by mixing with an authentic sample of methyl echinocystate received from Dr. C. R. Noller. $[\alpha]_D^{25} + 29°$ (chloroform).

Diacetate Methyl Ester.—The elviragenin diacetates were methylated with diazomethane, the product was recrystallized three times from methanol, m. p. 196–198°, which was not depressed when mixed with authentic methyl echinocystate diacetate.

SUMMARY

1. A toxic sapogenin was isolated from *Elvira biflora* (*Compositae*).

2. Hydrolysis yielded a sapogenin and five sugars.

3. Four sugars were identified as galactose, xylose, arabinose, and rhamnose.

4. The sapogenin was purified and some physical constants of the compound and three derivates were determined. The sapogenin was identified as echinocystic acid.

5. Some pharmacological tests were done with crude saponin and saponin purified by paper chromatography.

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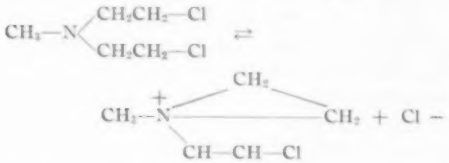
Polarographic Determination of the Rates of Formation of Ethyleneimonium Ions of Several Nitrogen Mustard Compounds

By HAROLD ZALLEN†, JOHN E. CHRISTIAN, and ADELBERT M. KNEVEL

The rates of formation of ethyleneimonium ion resulting only from the initial cyclization of a β-chloroethyl group of various nitrogen mustard compounds are reported. A polarographic technique was used for the quantitative determination of the ethyleneimonium ions. The nitrogen mustards investigated were both aliphatic and aromatic and contained two β-chloroethyl groups.

COMPOUNDS GENERALLY referred to as nitrogen mustards have been used to some extent as cancer chemotherapeutic agents. The pharmacological activity of these compounds is believed to result from a combination of two actions: (*a*) the internal cyclization of a β-chloroethyl group to form the highly reactive ethyleneimonium ion, hereafter referred to as the EI ion, and (*b*) the reaction of EI ion with various physiologically active groups within the body (1–4).

The initial cyclization process of a nitrogen mustard compound such as methyl-bis (β-chloroethyl) amine is shown in the following equation:



In dilute aqueous solutions at near neutral pH the reaction practically proceeds to completion before cyclization of the second β-chloroethyl group occurs.

Evidence of the presence of the EI ion was established by two methods (5). The first was the quantitative polarographic measurement (anodic) of thiosulfate uptake by EI ion. For each compound reported in this study, thiosulfate uptake indicated the formation of the ethyleneimonium-thiosulfate addition product. The second method involved the measurement of the diffusion current as obtained from the cathodic wave for the reduction of EI ion at the dropping mercury electrode.

The kinetic data presented in this paper is concerned only with rates of formation of EI ion resulting from the initial cyclization of a β-chloroethyl group of the nitrogen mustard compounds. These data were obtained by measuring the diffusion current of the EI ion at a constant potential and temperature with time.

The method of Kivalo, *et al.* (6), was used to determine the irreversibility of the EI ion waves. During the study it was also noted that $E^1/_2$ values changed to more negative potentials with an increase in pH. This phenomenon suggests an irreversible reaction.

The rates of formation of EI ion resulting from the initial cyclization of a β-chloroethyl group of various nitrogen mustard compounds were obtained for both aliphatic and aromatic nitrogen mustards containing one or two β-chloroethyl groups.

EXPERIMENTAL

Materials and Apparatus.—A Sargent model XXI pen type, potentiometric, recording polarograph provided with a dropping mercury electrode (D.M.E.) was used throughout this study. A modified method of Meites (7) Findeis and De Vries (8) was used to degas the test solutions. Triton-X-100[1] was used in a 0.002% concentration as the maximum suppressor. Analytical grade reagents were used throughout this study. Borate buffer solutions were prepared from stock solutions of 0.1 *M* sodium borate and 0.4 *M* boric acid. The capillary used throughout this study had a $m^{2/3}\,t^{1/6}$ value of 1.74 and was determined in a borate buffer solution, pH 7.36, at −0.20 volt *vs.* the saturated calomel electrode (S.C.E.). Mercury was purified by use of an "Oxifier."[2]

Polarographic Determination of EI Ions in Buffered Aqueous Solutions of Nitrogen Mustard Compounds.—The following procedure applies to each of the compounds investigated. All solvents used in this investigation were first equilibrated to 25 ± 0.01° in a Sargent Thermonitor water bath for one to three hours. A stock solution of 5×10^{-3} *M* was used for each nitrogen mustard compound studied. This solution was prepared by placing a calculated quantity of the compound in a

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† Present address: Auburn University, School of Pharmacy, Auburn, Ala.

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[1] Rohm and Haas Co., Philadelphia, Pa.

[2] Bethlehem Apparatus Co., Bethlehem, Pa.

TABLE I.—RATE OF CYCLIZATION AND HALF-WAVE POTENTIAL VALUES OF NITROGEN MUSTARD COMPOUNDS[a] IN BORATE BUFFER, pH 7.36, AT 25 ± 0.01°

2,2′-Dichlorodiethylamine HCl. No. 1[b]			2,2′-Dichloro-N-methyldiethylamine, N-oxide HCl. No. 5[b]			N,N-Bis(β-chloroethyl) glycine HCl. No. 551[b]			5-Chloro-N-(2-chloroethyl)-1-(chloromethyl)-pentylamine HCl. No. 33[b]			5-[Bis(2-chloroethyl)-aminomethyl]-4-(methoxymethyl)-2-methyl-3-pyridinol dihydrochloride. No. 619[b]		
Time, min.	i_t, μa.	$i_\infty - i_t$, μa.	Time, min.	i_t, μa.	$i_\infty - i_t$, μa.	Time min.	i_t, μa.	$i_\infty - i_t$, μa.	Time, min.	i_t, μa.	$i_\infty - i_t$, μa.	Time, min.	i_t, μa.	$i_\infty - i_t$, μa.
0	1.76	1.75	0	3.26	2.70	0	2.20	4.79	0	2.10	3.50	0	1.68	1.52
50	2.06	1.45	2	4.14	1.82	1	4.34	2.65	60	3.02	2.58	2	2.24	0.96
100	2.37	1.14	4	4.69	1.27	2	5.50	1.49	120	4.55	1.05	4	2.60	0.60
150	2.52	0.99	6	5.10	0.86	3	6.17	0.82	180	5.01	0.59	6	2.82	0.38
200	2.68	0.83	8	5.37	0.59	4	6.53	0.46	240	5.27	0.33	8	2.96	0.24
250	2.83	0.68	10	5.55	0.41	5	6.73	0.26	300	5.42	0.18	10	3.05	0.15
300	2.94	0.57	12	5.69	0.27	6	6.85	0.14	360	5.50	0.10	12	3.11	0.09
350	3.04	0.47	14	5.77	0.19	∞	6.99		∞	5.60		∞	3.20	
∞	3.51	...	∞	5.96										
k[c] 0.0033 min.$^{-1}$ $E_{1/2}$ −1.83 v.			k 0.187 min.$^{-1}$ $E_{1/2}$ −1.80 v.			k 0.582 min.$^{-1}$ $E_{1/2}$ −1.81 v.			k 0.0092 min.$^{-1}$ $E_{1/2}$ −1.52 v.			k 0.237 min.$^{-1}$ $E_{1/2}$ −1.81 v.		

[a] Structural formulas and other data available (9). [b] The number following each compound in the table corresponds to the number found in the tables of ref. 9. [c] The specific rate constant, k, was calculated graphically; all kinetic studies were run in duplicate; respective k values agreed within experimental error.

100-ml. volumetric flask and adding borate buffer, pH 7.36, to the mark. All compounds were aged for one hour except 5-[bis(2-chloroethyl)aminomethyl]-4-(methoxymethyl)-2-methyl-3-pyridinol dihydrochloride and 5-chloro-N-(2-chloroethyl)-1-(chloromethyl)pentylamine hydrochloride which were aged for two hours prior to their analyses. At the end of the aging period, aliquots of 5×10^{-3} *M* solution of the nitrogen mustard ranging from 2 to 15 ml. were each transferred to 50-ml. volumetric flasks each containing from 15 to 25 ml. of borate buffer and 0.002% Triton-X-100, so when diluted to the mark the solution had a pH of 7.37. Polarograms were made over a potential span of 0.00 to −2.00 volts *vs.* S.C.E. The values of the half-wave potentials for the various EI ions polarographed are reported in Table I.

Kinetic Studies.—Kinetic studies were carried out in a borate buffer at pH 7.36 at 25° ± 0.01°. A modified Lingane-Laitinen H-type cell was used throughout these studies. All solvents were first equilibrated to the stated temperature prior to use. The increase in concentration of the EI ion with time was followed polarographically by imposing a constant applied potential of −1.50 volts *vs.* S.C.E. This potential corresponds to the plateau region of the EI ion investigated.

About 250 ml. of borate buffer, pH 7.36, was degassed for one hour in a degassing flask immersed in the constant temperature bath. The previously weighed nitrogen mustard under investigation (5×10^{-2} *M*) was placed in a 50-ml. volumetric flask and filled to the mark with degassed borate buffer. Five to 15-ml. aliquots were used for each kinetic study. The analysis was initiated at exactly the same time (zero time) the aliquot was placed in the cell. The reaction was blanketed with nitrogen during the kinetic study and was considered complete when a constant diffusion current was reached. All diffusion current readings were corrected for residual current. Specific rate constants were determined from the slope of the plot of log $(i_\infty - i_t)$ *vs.* time, where i_∞ represents the diffusion current when the reaction was considered complete for all practical purposes, and i_t represents the diffusion current at time, t. Experimental data are reported in Table I.

SUMMARY

1. A polarographic procedure has been utilized to determine the rates of formation of EI ion resulting only from the initial cyclization of a β-chloroethyl group of some aliphatic and aromatic nitrogen mustard compounds.

2. The rate of formation of EI ion for each nitrogen mustard compound investigated in this study followed first-order kinetics.

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Simple Rheometer for Definition of Viscoelastic Properties of Structural Liquids

By DUNCAN E. McVEAN and ALBERT M. MATTOCKS

A "rising sphere" instrument is described which records the stress as a function of strain imposed on a liquid by a sphere being pulled upwards. Measurements involve only strain of the liquid structure and not flow. Relaxation constants and other elastic constants are derived via the typical mechanical models for viscoelasticity. The results are believed to be particularly valuable to the study of sedimentation of suspensions. Examples of data for several structural systems are presented. The apparatus is simple and inexpensive to construct.

Since Bingham's plastic flow was first described, there has been concern over the concept of yield value. The yield value has been simply described as a fixed tangential force which must be exceeded before flow occurs between two layers of a plastic liquid or gel, force above this minimum acting in the usual manner to cause proportional shear (1). This is expressed in the Bingham equation for plastic flow

$$F = f + US \qquad \text{[Eq. 1]}$$

where F is stress, f is the yield value, S is shear, and U is the coefficient of plastic viscosity. Numerous authors have agreed with this concept (2, 3), although the actual existence of a yield value has been questioned (1). With the usual type of viscometer a yield value is not observed directly but obtained by extrapolation of the flow curve to zero shear rate. Instruments have been designed, however, such as that of Pryce-Jones (4) and of McDowell and Usher (4) which measure residual stress on a bob after shear has been stopped.

The ideal plastic, following Eq. 1, is seldom encountered, but the more common flow curve thought to exhibit yield value is one which is continuously curved throughout, as illustrated in Fig. 1. This makes graphical extrapolation difficult, and other methods have been used to estimate the yield value. Meyer and Cohen (5), for example, extrapolated a line drawn through stress-shear points at the two lowest rates of shear measured to zero shear, while Grim, *et al.* (6), used a flow equation which allowed calculation of stress at zero rate of shear. These estimates of a yield value may be useful in application, as pointed out by Alfrey (1), but they often do not agree with the recorded flow curves. Even with consideration of the plug flow region (7) laminar flow appears to exist at values of stress below the estimated yield value; thus, one may question the existence of a measureable fixed yield value. A more acceptable concept would be that of Alfrey (1), in which the region normally called yield value is considered to represent a rapid change in the order of flow and not a sudden change from zero to finite flow.

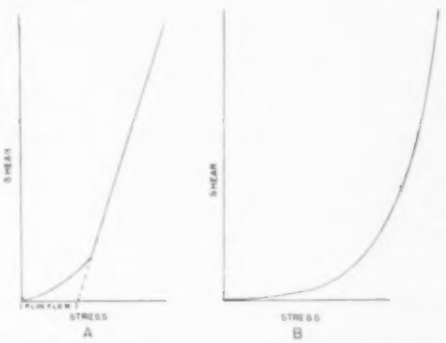

Fig. 1.—Curves illustrating ideal plastic flow, *A*; and more commonly encountered curves where yield value may be thought to exist, *B*.

The concept of yield value is important to the field of pharmacy, since the resistance to flow of a suspension at low shearing stress may be utilized to form permanent suspensions. This approach was demonstrated by Meyer and Cohen (5) who prepared permanent suspensions of marbles, golf balls, and sand. This need for a clarification of the nature of yield value and the desire to obtain a method for its direct measurement stimulated the design of the instrument reported in this paper.

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EXPERIMENTAL

Description of the Rheometer.—A diagram of the rising sphere rheometer is given in Fig. 2. It consists essentially of a steel sphere attached through a strain gauge to an arm which can be raised at extremely slow rates. In this manner the ball can be used to strain the gel below the limit at which flow occurs with constant recording of the corresponding stress. With the present equipment eight rates of strain can be used: 8.52×10^{-1}, 6.18×10^{-1}, 4.28×10^{-1}, 3.40×10^{-1}, 5.9×10^{-2}, 4.7×10^{-2}, 3.6×10^{-2}, 3.4×10^{-2}, sec.$^{-1}$.

The rate of shear or rate of strain for this instrument is calculated from Stoke's equation for the falling sphere (8)

$$F = 3\pi \mu VD \qquad \text{[Eq. 2]}$$

where F is stress, D is the diameter of the sphere, V is the velocity of the sphere, and μ is the dynamic viscosity. Rate of shear, S, then is equal to

$$S = F/\mu = 3\pi VD \qquad \text{[Eq. 3]}$$

and for this instrument:

$$S = 17.907\ V \qquad \text{[Eq. 4}$$

Stress on the sphere, in dynes/cm.^{2}, is obtained by dividing the observed resistance in dynes by the surface area of the sphere. The basis for use of surface area was presented by Rouse and Howe (8). A formula for obtaining the average stress on the liquid between the surface of the sphere and the wall of the vessel was devised and tested by Bacon (9). Using this gives the folowing equation for average stress, F, with the rising sphere viscometer

$$F = F_{obs}\,[1\text{–}2.104\,d/D + 2.09\,(d/D)^3 - 0.95\,(d/D)^5] \qquad \text{[Eq. 5]}$$

where F_{obs} is the force reading of the strain gauge, d is the diameter of the sphere, and D is the diameter of the vessel containing the liquid. With a sphere of 1.90 cm. and cylinder of 9.6 cm., the factor within the brackets of Eq. 5 is 0.6910.

The rate of shear induced in a liquid without structure is so small that no recording of shearing stress can be measured; for example, no measurement of stress was recorded at any of the rates of shear listed above when the sphere was immersed in undiluted glycerin or syrup. Thus, the forces measured may be considered to be only the viscoelastic resistance of the gel structure and not shearing stress resulting from viscous flow.

Results Obtained with the Rheometer.—Several systems were prepared which were known to possess "yield value." Cetyl alcohol emulsions were made from the following formula

Methylparaben	2.40 Gm.
Propylparaben	1.44 Gm.
Sodium lauryl sulfate	24.00 Gm.
Cetyl alcohol	320.00 Gm.
Water	3,376.00 Gm.

The parabens and sodium lauryl sulfate were dissolved in the water at 50°, and the cetyl alcohol was added with stirring at the same temperature. The mixture was passed three times through a preheated homogenizer at 1,000 lb. pressure and stirred as it cooled to room temperature. The emulsion was then passed through a colloid mill three times and allowed to stand at room temperature until measured.

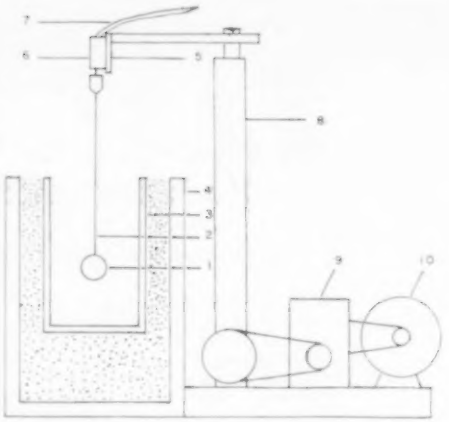

Fig. 2.—The rising sphere rheometer. 1, stainless steel sphere, 1.90 cm. diam.; 2, platinum-rhodium wire, 36 gauge; 3, glass vessel, 9.6 cm.diam.; 4, thermostated water bath; 5, support arm; 6, Statham transducer, Gl-1.5-300, Statham Instruments, Hato Rey, Puerto Rico; 7, wire leading to amplifier and recorder; 8, Gardner tensile strength apparatus, Gardner Laboratory, Bethesda, Md.; 9, Gear reduction unit 1:50; 10, motor.

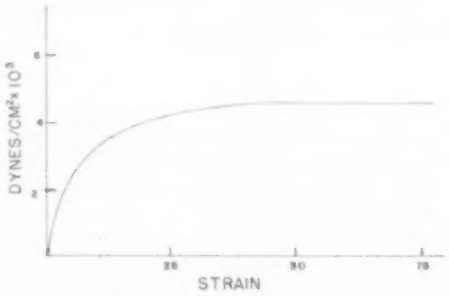

Fig. 3.—Typical upcurve with rising sphere rheometer. Point of flow is seen as beginning of horizontal line.

A sol of Carbopol 934 was prepared by dispersing 10 Gm. of the polymer in 2 L. of water with a high-speed mixer, allowing the mixture to stand for an hour, then neutralizing with 10% sodium hydroxide, using gentle mixing.

A Veegum HV suspension was prepared by dispersing 20 Gm. of the dry powder in 1.8 L. of water, allowing it to stand for forty-eight hours, then diluting it to 2 L. with water.

As the sphere was drawn continuously through a liquid with "yield value," a curve such as shown in Fig. 3 was obtained. An increasing stress was recorded until the point at which flow began, and beyond that point the stress became constant. This general type of curve was obtained with all structural liquids, although the first portion of the curve, approaching the flow point of yield value,

TABLE I.—CONSTANTS OF THE RELAXATION EQUATION FOR CARBOPOL 934 SUSPENSION AT THREE LEVELS OF STRAIN

Strain	A Dynes/cm.²	B Dynes/cm.²	C Dynes/cm.²	α sec.$^{-1}$ × 10^3	β sec.$^{-1}$ × 10^4	γ sec.$^{-1}$ × 10^5
21.25	1088.7	694.2	2247.2	1.04	10.91	3.00
12.10	90.5	131.5	363.5	0.93	1.25	1.94
0.61	60.1	95.3	185.1	1.77	3.83	0.67

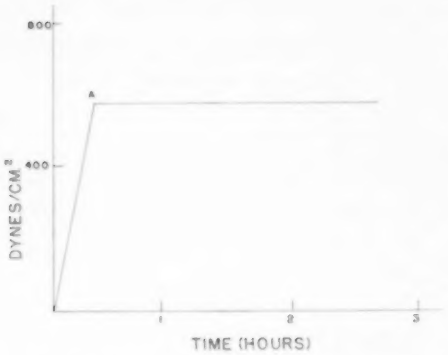

Fig. 4.—Rheometer upcurve for Carbopol 934. At point *A* strain was no longer increased but held constant.

Fig. 5.—Relaxation curves for cetyl alcohol emulsion at two levels of constant strain.

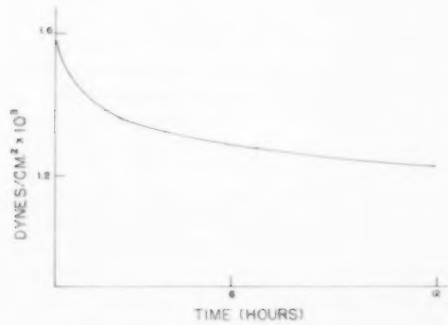

Fig. 6.—Relaxation curve for Veegum HV suspension.

was curved in some instances, linear in others. The observed flow point varied with change in rate of rise of the sphere, indicating that the yield value is a function of rate of strain.

With the Carbopol 934 sol it was found that the straining could be stopped at any point and the stress would remain constant as long as the strain was unchanged. This is illustrated in Fig. 4. It should be noted, also, that the upcurve for this material is linear.

With cetyl alcohol emulsion, relaxation of stress occurred at any point where straining was stopped. The relaxation of stress at two levels of constant strain is illustrated in Fig. 5.

The Veegum HV dispersion showed relaxation of stress much like that of cetyl alcohol emulsion, as seen in Fig. 6.

DISCUSSION

The curve for Carbopol 934 is typical for an ideal elastic body for which stress is proportional to strain and no relaxation occurs. The slope of the upcurve relating stress to strain for such a body is the shear modulus, G

$$G = F/s, \qquad \text{[Eq. 6]}$$

where F is stress and s is strain. For the dispersion tested the shear modulus was found to be 75.0 dynes/cm.². A structural liquid with ideal elasticity, such as this, would appear to be the best type for obtaining permanent suspension, since it would continue *ad infinitum* to resist the deformation by a falling particle.

The relaxation of stress at constant strain observed with cetyl alcohol emulsion and Veegum HV dispersion is typical of bodies with both retarded and instantaneous elasticity, represented by a model containing three Maxwell elements in parallel (10). The equation for this type of system is

$$F = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t} \qquad \text{[Eq. 7]}$$

where F is stress at constant strain, A, B, and C are products of strain and shear moduli, constant at a given level of strain, and α, β, and γ are constants equivalent to the reciprocals of Maxwell relaxation times of the elements of the model.

The constants, α, β, and γ, should be constant, according to theory, regardless of the state of strain at which they are measured. Values of these constants for the cetyl alcohol emulsion at three different levels are presented in Table I, where it is seen that some variation was found. Whether these differences are due to experimental errors of evaluation is not yet clear, and whether one might expect these to be true constants is still questionable, since the three-element model is an artifact used to represent a distribution of molecules or particles and not a group of three distinct categories. The

answers to these questions must await application to a number of different systems with a detailed evaluation of experimental errors involved.

The authors hesitate to publish values for the constants of the relaxation equation for Veegum HV, since this system is thixotropic and constants obtained are a function of the history of the particular suspension studied.

The rising sphere viscometer is a simple instrument which appears promising as a tool for the direct measurement of yield value and for determining the nature of yield value in different systems, and it is expected that knowledge so gained will be of value in understanding the phenomena of sedimentation in pharmaceutical suspensions.

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Approximate Rates of Shear Encountered in Some Pharmaceutical Processes

By NORMAN L. HENDERSON†, PAUL M. MEER‡, and H. B. KOSTENBAUDER

Approximate values are presented for rates of shear encountered in the manufacture, handling, and application of some pharmaceutical liquids and semisolids. Examples considered are milling, flow of liquids from bottles, through tubes and hypodermic needles, and the topical application of liquids and semisolids. The importance of this information for the effective utilization of rheological data in the development, control, and packaging of non-Newtonian pharmaceuticals is indicated.

RHEOLOGY has become an accepted tool in the development and control laboratories of the pharmaceutical industry, and many excellent discussions of the theory of flow and the characteristics of Newtonian and non-Newtonian materials, including pharmaceuticals, have been published (1–7). Although instruments and procedures for obtaining rheological data in fundamental units of rate of shear and shearing stress have also been described in some detail (1, 2, 7–10), relatively little information has been published concerning the actual interpretation and application of rheological data in the development, processing, and control of pharmaceutical products by correlating rheological data product performance.

Such correlations are especially difficult when it becomes necessary to relate performance to flow curves of rheologically different materials. The parameters generally used to describe the flow properties of Newtonian and non-Newtonian liquids (viscosity, plastic viscosity, yield value, apparent viscosity, degree of thixotropic breakdown) are essential for theoretical considerations of the influence of variables on flow properties and for such engineering problems as calculation of pressure losses along pipe lines, but the task of applying such involved treatment to a process as variable as that of pouring a liquid from a bottle is a foreboding one. In cases such as this it may be helpful to consider just what qualities of the product are to be evaluated with these rheological data. In a spreadability problem, for example, irrespective of the rheological classification of the material being subjected to manipulation, the factor which is of interest is the resistance which the material offers when being spread at a specified rate. This process of spreading is exactly analogous to the process of shearing the material in a rotational viscometer. The shearing stress (τ) as measured by the rotational viscometer is then a direct indication of the resist-

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† Merck Sharp & Dohme Fellow.
‡ Present address: Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

ance which the product offers to spreading. The shearing stress will of course vary with the rate of shear, but if it is possible to approximate the shearing rate involved in the process it is then a simple matter to look at the consistency curve and obtain the shearing stress directly at the appropriate rate of shear. Shearing rates for processes such as this can be readily approximated as indicated previously by Kostenbauder and Martin (8).

It should be possible to extend this treatment to other processes which are primarily shearing processes. Thus, manipulating an ointment on an ointment tile, or passing it through a roller mill would be primarily processes in which the product is subjected to shearing effects, and the resistance of the ointment to the shearing would be the shearing stress (τ), as measured by the rotational viscometer at the appropriate rate of shear.

Flow of a liquid in a narrow tube, at a velocity below that producing turbulence, results in a laminar flow and permits approximation of a mean rate of shear for the liquid. An example might be a liquid flowing through a hypodermic needle or through a tube such as that found in an aerosol package. The force required to cause a liquid to flow through such a tube should be related to the shearing stress at the rate of shear to which the liquid is subjected in each specific process.

Not all processes can be expected to be so amenable to this treatment. Flow of a liquid from a bottle can probably be predicted only somewhat qualitatively, and a process such as squeezing an ointment from a tube, which is not a shearing process, might not be expected to correlate at all with viscometry data.

In this paper methods are described for obtaining approximate values for shearing rates involving several processes to which pharmaceuticals might be subjected. In making these approximations Newtonian behavior is assumed for calculation of shearing rates. While this of course cannot be so, the errors so introduced are probably not greater than the variability of the process being considered.

EXPERIMENTAL

Spreadability.—Kostenbauder and Martin (8) illustrated the approximation of rate of shear in the spreading of an ointment on a surface by application of the equation for rate of shear

$$S = dv/dr \qquad \text{(Eq. 1)}$$

where v is the velocity at which the ointment is being sheared and r is the thickness of the layer. Assuming that in applying an ointment to the skin a stroke averaging 6 cm. in length is used, at a rate of about four strokes per second, and the applied layer is 1–3 mm. thick, the rate of shear, S, can be approximated as follows. For this very small clearance the expression may be written as

$$S = \Delta v/\Delta r \qquad \text{(Eq. 2)}$$

The velocity of the top layer of ointment would be 24 cm./sec., and the layer in contact with the skin would be stationary; Δr would simply be the thickness of the layer.

$$S = \frac{24 \text{ cm./sec.}}{0.2 \text{ cm.}} = 120 \text{ sec.}^{-1} \qquad \text{(Eq. 3)}$$

Any method which is designed to evaluate the spreadability of a topical preparation should be designed to produce a rate of shear approximating that expected to be encountered in application of the product. The value of 120 sec.$^{-1}$ is not presented as a specific reference point; different types of preparations might well be applied at entirely different rates of shear, but these can be readily approximated.

Several spreadability determinations were made with an apparatus similar to that described by Mutimer, *et al.* (11). The general handling of samples was similar to that indicated by Mutimer, and the results are presented in Fig. 1. It is of interest that the maximum rate of shear encountered in the use of this apparatus was about 10 sec.$^{-1}$. At this low rate of shear there appears to be considerable similarity in spreadability of zinc oxide ointment U.S.P. XV and hydrophilic ointment U.S.P. XV;

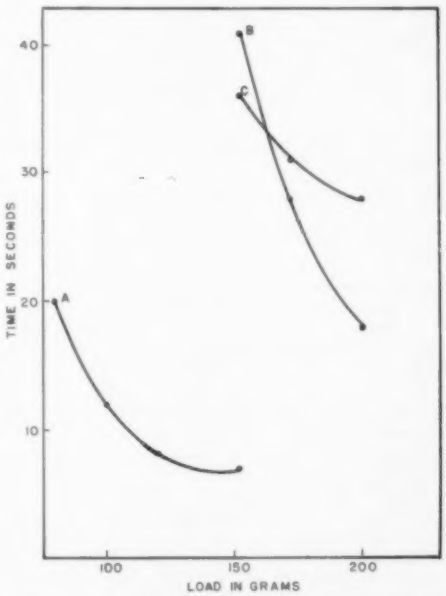

Fig. 1.—Spreadability curves obtained with an apparatus similar to that described by Mutimer, *et al.* (11). Graphs show time required for a slide to move 6 cm. under indicated driving load in grams. *A*, Petrolatum U.S.P. XV, B, zinc oxide ointment U.S.P. XV, C, hydrophilic ointment U.S.P. XV.

however, as shown by the consistency curve in Fig. 2, the two ointments would show considerably different spreading characteristics if applied at a shearing rate of 120 sec.$^{-1}$.

Milling.—Approximate rates of shear encountered in the use of a roller mill can be obtained in a manner quite analogous to that described for spreading a topical preparation. In this case, Δv would be the difference between roll speeds of the two faster moving surfaces, and Δr would be the clearance between rolls. A typical example might be roll speeds of 167 cm./sec. and 55 cm./sec., with a clearance of 0.009 cm.

$$S = \frac{167 \text{ cm./sec.} - 55 \text{ cm./sec.}}{0.009 \text{ cm.}} = 12{,}333 \text{ sec.}^{-1} \quad \text{(Eq. 4)}$$

Both shearing and compression might be encountered in such a milling operation, but the calculated shearing rates indicate that shearing undoubtedly is a quite important factor.

Calculations of this nature applied to colloid mills give results of several hundred thousand reciprocal seconds.

Hypodermic Needles and Small Tubes.—The velocity distribution for flow of a Newtonian liquid through a tube is parabolic in form (12). Since the volume of a paraboloid of revolution is one-half that of the circumscribing cylinder, the mean velocity of the cylinder of fluid will be one-half the maximum velocity for the center of the paraboloid. If a liquid is found to flow through a hypodermic needle of 0.05-cm. radius at a rate of 2 ml. per second, the rate of shear might be estimated as follows:

$$\text{volume of cylinder} = \pi r^2 h \quad \text{(Eq. 5)}$$

$$\text{volume/sec.} = (3.14)(0.05 \text{ cm.})^2 h = 2 \text{ ml./sec.} \quad \text{(Eq. 6)}$$

$$h = 237 \text{ cm./sec. (mean velocity of cylinder of fluid)} \quad \text{(Eq. 7)}$$

Maximum velocity for fluid at center of paraboloid is then 2×237 cm./sec. = 474 cm./sec.

$$S = dv/dr = \text{maximum velocity}/r \quad \text{(Eq. 8)}$$

$$S = \frac{474 \text{ cm./sec.}}{0.05 \text{ cm.}} = 9{,}480 \text{ sec.}^{-1} \quad \text{(Eq. 9)}$$

Thus, one might expect to encounter shearing rates of thousands of reciprocal seconds in the use of injectable materials, and evaluations of flow properties should be made at these very high shearing rates.

A similar approximation made for a typical nasal spray packaged in a plastic squeeze bottle provided an approximate rate of shear of 2,000 sec.$^{-1}$. Approximations of this type might also be applied to high speed filling equipment.

Figure 3 illustrates consistency curves for glycerin and for a Carbopol[1] dispersion. Figure 4 shows estimated shearing rates for the flow of these two liquids through a 6-mm. tube under varying driving pressures. Qualitatively, at least, this graph is in agreement with predictions which might be made from inspection of the consistency curve shown in Fig. 3.; the Carbopol dispersion requiring greater driving pressure at very low rates of shear, while the glycerin exhibited greater resistance to flow at higher rates of shear. Knowledge of the approximate rate of shear to be encountered in application would permit selection of the most appropriate medium.

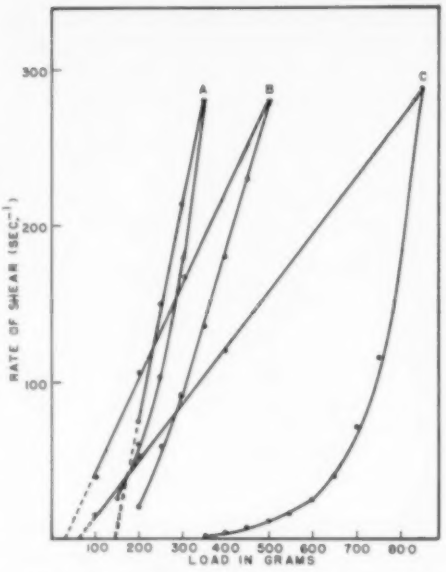

Fig. 2.—Consistency curves obtained with the modified Stormer viscometer described by Kostenbauder and Martin (8). A, Petrolatum U.S.P. XV, B, zinc oxide ointment U.S.P. XV, C, hydrophilic ointment U.S.P. XV.

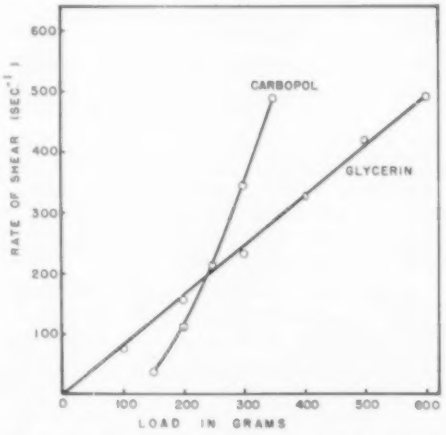

Fig. 3.—Consistency curves for U.S.P. glycerin and a 0.25% aqueous solution of Carbopol 934, 31°. A Stormer viscometer was employed with a cup and bob similar to that described by Fischer (14).

Pouring from a Bottle.—It seems reasonable to treat flow from a bottle as occurring through one-half of the area of the mouth of the bottle. The flow is then similar to flow in an open channel, and

[1] Carbopol 934, B. F. Goodrich Chemical Co., Cleveland, Ohio, is a carboxy vinyl polymer.

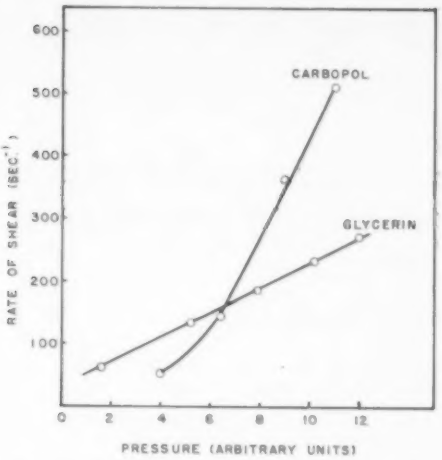

Fig. 4.—Rate of flow of U.S.P. glycerin and a 0.25% aqueous solution of Carbopol 934 through a 6-mm. glass tube under varying driving pressure (air pressure).

the geometry of flow through the bottle neck can be considered to be one-half of a right circular cone. The following is a sample calculation for the rate of shear involved in flow of glycerin poured from a 16-oz. bottle with a neck radius of 0.91 cm. at a rate of 21 ml./sec.

$$^1/_2 \text{ volume of rt. circular cone} = {}^1/_2 ({}^1/_3 \pi r^2 h) = 21 \text{ ml./sec.} \quad \text{(Eq. 10)}$$

$$h = 48.5 \text{ cm./sec. (maximum velocity)} \quad \text{(Eq. 11)}$$

$$S = dv/dr = \frac{48.5 \text{ cm./sec.}}{0.91 \text{ cm.}} = 53.3 \text{ sec.}^{-1} \quad \text{(Eq. 12)}$$

Observations on the pourability of the two fluids represented in Fig. 3 indicate that the estimated rate of shear for pouring is reasonable. While glycerin was readily poured from the 16-oz. bottle, the Carbopol dispersion did not pour at a practical rate, as might be indicated at least qualitatively from the consistency curve.

Maximum values of about 90 sec.$^{-1}$ were obtained for rates of shear involved in pouring very fluid suspensions such as calamine lotion, and minimum values of about 10 sec.$^{-1}$ were observed for a series of commercial suspensions. Most suspensions investigated gave estimated shearing rates of 20–30 sec.$^{-1}$ when poured from their original containers. These values may not be particularly meaningful, however, particularly for suspensions showing large yield values.

The size of the container did not appear to change the rate of shear as much as might be expected, although suspensions packaged in small containers may be quite difficult to pour as a result of the decreased mass of fluid and therefore a decreased driving force.

Extrusion from Tubes.—Extrusion of soft solids is not primarily a shearing process, but one of compression (13). Observations on a number of ointment bases and comparison with consistency curves indicated that there was no obvious correlation between the rheograms and extrusion pressure.

SUMMARY

The following rates of shear have been estimated for some typical pharmaceutical processes: topical application, up to 120 sec.$^{-1}$; rubbing on ointment tile, 150 sec.$^{-1}$; roller mill, 1,000–12,000 sec.$^{-1}$; colloid mill, several hundred thousand sec.$^{-1}$; hypodermic needle, up to 10,000 sec.$^{-1}$; nasal spray (plastic squeeze bottle), 2,000 sec.$^{-1}$; and pouring from bottle, below 100 sec.$^{-1}$.

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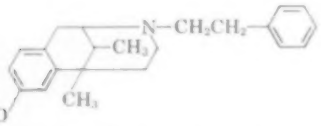
Phenazocine Hydrobromide: Pharmaceutical Aspects of a New Benzomorphan Analgesic Related to Morphine

By W. E. LATSHAW and D. R. MacDONNELL

Some chemical and physical properties of phenazocine hydrobromide are presented, including X-ray diffraction data and infrared curves on three known crystalline forms. A schematic representation of a method of synthesis is included. Several color reactions and microchemical tests are given which can be used to differentiate phenazocine from codeine salts, morphine salts, and meperidine hydrochloride. Formulas for clinically acceptable ampuls and multiple-dose vials are presented along with stability data on these dosage forms.

IN THEIR SEARCH for a new analgesic of high potency and low addictive liability, May and Eddy synthesized phenazocine (NIH 7519). This drug was found to produce profound analgesia and was considered to be "a promising agent for the relief of both acute and chronic pain" (1).



Phenazocine; 1,2,3,4,5,6-hexahydro-8-hydroxy-6,11-dimethyl-3-phenethyl-2,6-methano-3-benzazocine

A pharmacologic and clinical evaluation of phenazocine[1] (NIH 7519) has proved it to be an effective analgesic with similar but less limiting liabilities than those of other narcotic analgesics currently in use (2–4). It has been employed successfully in general pain states, as preoperative medication, as an anesthetic adjunct, in postoperative restlessness and pain, and in obstetrics (2, 3).

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The authors are indebted to Dr. Eugene DeWald for supplying the infrared curves. Measurements shown in Table I were made by Dr. Walter E. Thompson using X-ray diffraction patterns kindly supplied by the Department of Chemical Control, Mallinckrodt Chemical Works. The authors are also grateful for the assistance of Mr. J. Cannon and Mrs. B. Rizzo in various phases of this study.

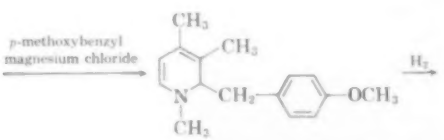
[1] Registered trade name of Smith Kline & French is Prinadol.

EXPERIMENTAL

Synthesis

A schematic representation of a method for the synthesis of phenazocine hydrobromide is as follows:

3,4-lutidine — methyl iodide — 3,4-lutidine methiodide



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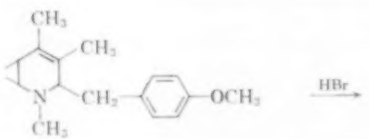

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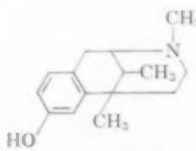
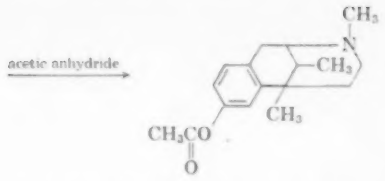


1,2,3,4,5,6-hexahydro-8-hydroxy-3,6,11-trimethyl-2,6-methano-3-benzazocine



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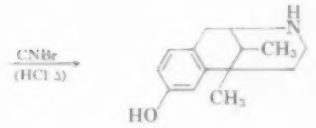

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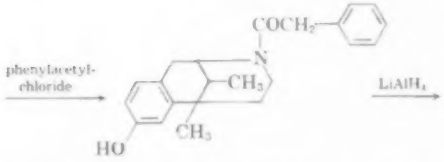
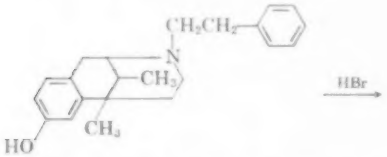
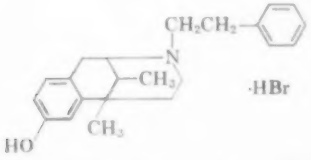


1,2,3,4,5,6-hexahydro-8-hydroxy-3-phenylacetyl-6,11-dimethyl-2,6-methano-3-benzazocine



1,2,3,4,5,6-hexahydro-8-hydroxy-3-phenethyl-6,11-dimethyl-2,6-methano-3-benzazocine



1,2,3,4,5,6-hexahydro-8-hydroxy-3-phenethyl-6,11-dimethyl-2,6-methano-3-benzazocine hydrobromide (phenazocine hydrobromide)

Chemical and Physical Properties

Phenazocine is utilized as the racemic hydrobromide salt, this salt being readily obtained from the final reaction mixture. It is not particularly sensitive to heat, light, or air. Aqueous solutions are partially decomposed when autoclaved unless protected by buffering at an acidic pH.

Phenazocine hydrobromide is a white, nonflowing, odorless, crystalline powder with a bitter taste, m.p. 165–170°, mol. wt. 402.39, pKb 3.8. It contains 79.9% phenazocine free base. The free base precipitates from aqueous solutions at approximately pH 6.2 when sodium or potassium hydroxide solutions are added, and will redissolve at a pH of 11.6. With solutions of ammonium hydroxide the free base is also precipitated out of aqueous solutions at approximately pH 6.2 but the precipitate does not redissolve upon adding an excess of ammonium hydroxide. A 0.5% aqueous solution of the hydrobromide salt has a pH of about 5.9. It is very soluble in propylene glycol, freely soluble in ethyl or methyl alcohol, only slightly soluble in water, sparingly soluble in chloroform or acetone, and practically insoluble in carbon tetrachloride. The water solubility of commercial material varies depending upon the amount of each polymorphic form present. The approximate water solubility is 0.5%, heat being required to effect solution in a reasonable period of time.

It has been found that the hydrobromide salt can exist in three polymorphic forms (A, B, and C). Infrared absorption spectrographs of each of these three forms are shown in Fig. 1.

Forms A and B are anhydrous whereas form C is a hemihydrate, containing 2.18% water. Commercial material is frequently a mixture of two or all three of the forms. Form C is obtained when phenazocine hydrobromide is crystallized from water. Recrystallization of form C from absolute alcohol may yield any one of the three forms. Crystallization of phenazocine hydrobromide from a strictly anhydrous system yields either form A or form B. Form B is metastable and is slowly converted to form A upon standing in the presence of the mother liquor. Form A appears as parallelogram-shaped plates, form B as six-sided plates, and form C as thick prisms (5).

The three crystalline forms of phenazocine hydrobromide give well defined X-ray diffraction patterns. Diffraction data on each of these forms (A, B, and C) are given in Table I.

Table I.—X-Ray Diffraction Data for Phenazocine Hydrobromide[a]

Form A		Form B		Form C	
d (Å.)	I/I max.	d (Å.)	I/I max.	d (Å.)	I/I max.
16.05	15	15.22	40	14.72	5
8.62	10	13.38	15	8.84	40
7.49	5	10.52	10	7.02	2
6.37	10	7.49	10	6.51	6
6.15	10	6.23	10	5.52	10
5.86	5	5.98	10	5.27	6
5.53	10	5.15	100	5.12	15
5.21	15	4.82	6	4.92	15
4.94	40	4.31	100	4.72	6
4.46	10	4.14	15	4.67	6
4.35	10	3.97	40	4.39	100
4.19	100	3.62	10	4.23	6
3.81	10	3.50	6	4.15	10
3.48	5	3.37	6	4.02	15
3.40	10	3.32	10	3.93	6
3.25	40	2.99	6	3.77	6
2.96	15	2.81	6	3.69	6
2.78	5	2.70	6	3.62	6
				3.50	5
				3.41	5
				3.33	25
				3.21	15
				3.11	5
				2.94	5

[a] Obtained with copper radiation (Kα) and nickel filter.

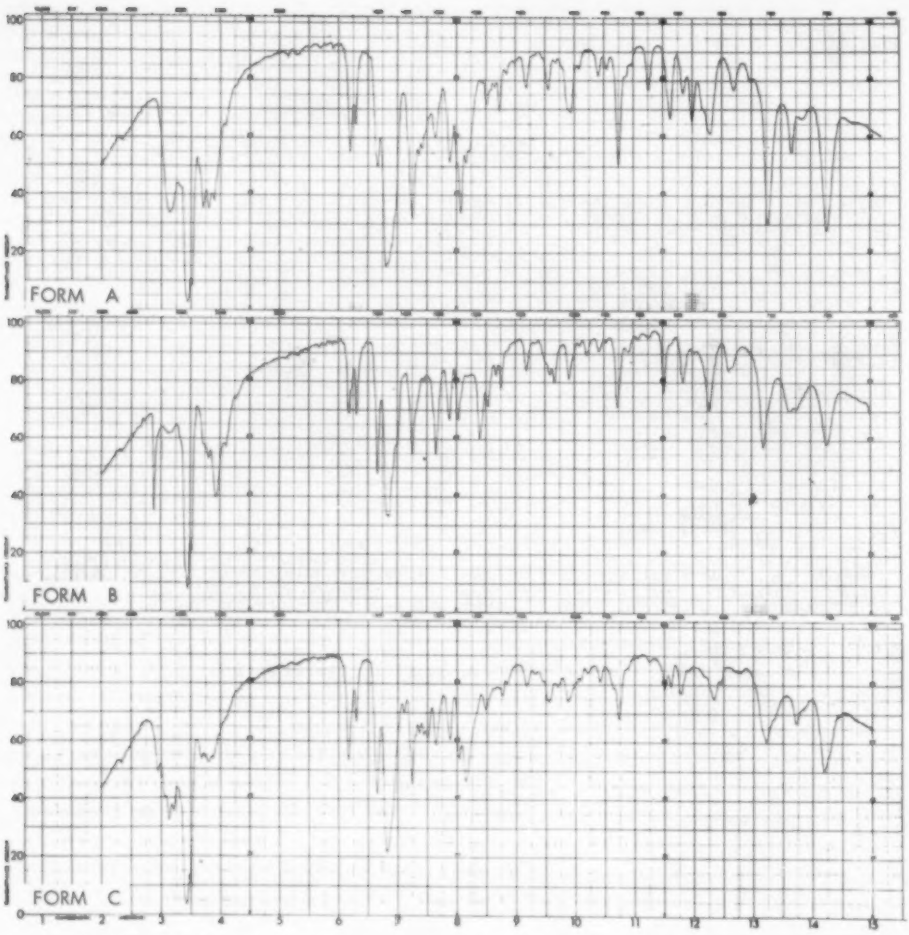


Fig. 1.—Infrared absorption spectrographs of three forms of phenazocine hydrobromide in Nujol.

A spectrophotometric assay method for purity and stability of the chemical and dosage forms, based on the absorption at 278 mμ, has been developed (6).

Identification

Color Reactions.—Two reagents have been found which will distinguish phenazocine hydrobromide from codeine salts, morphine salts, and meperidine hydrochloride. These reagents are aqueous sodium tungstate (1%) and *p*-dimethylaminobenzaldehyde (10% in glacial acetic acid).

Procedure.—Place a drop of solution to be tested (1 mg. drug per ml. of 1% acetic acid) on a white spot plate or small watch glass and add a drop of the reagent or test solution. Evaporate to dryness. Add to the residue a drop of concentrated sulfuric acid. The characteristic color changes are indicated in Table II.

Using a similar procedure it is also possible to differentiate commercial aqueous phenazocine hydrobromide solutions from aqueous solutions of codeine salts, morphine salts and meperidine hydrochloride.

Procedure.—Place a drop of the aqueous solution to be tested on a white spot plate or watch crystal and add a drop of the reagent or test solution. Evaporate to dryness. Add to the residue a drop of concentrated sulfuric acid. These color changes are listed in Table III. Other color tests for the identification of phenazocine have been noted by Clarke (7).

Precipitation Reactions.—Many alkaloidal reagents or test solutions give precipitates when added to 0.5% aqueous solutions of phenazocine hydrobromide, codeine salts, morphine salts, and meperidine hydrochloride. Two of these, palladium chloride and sodium nitroprusside, can be used to distinguish phenazocine hydrobromide from the other three narcotic drugs by the fact that it alone gives an amorphous precipitate as indicated in Table IV. Concentrations of phenazocine hydrobromide as low as 1 mg./ml. and 0.05 mg./ml. will react with palladium chloride and sodium nitroprusside, respectively, to produce a precipitate readily detectable to the naked eye. Penicillin G

TABLE II.—COLOR REACTIONS WHICH DISTINGUISH PHENAZOCINE HYDROBROMIDE FROM CODEINE SALTS, MEPERIDINE HYDROCHLORIDE, AND MORPHINE SALTS

Reagent or T. S.	Drug Solution, 1 mg./ml. 1% HAc	Color Reaction
Sodium Tungstate, (1% in H_2O)	Codeine SO_4	Light grayish-blue
	Codeine HCl	Light grayish-blue
	Codeine PO_4	Light grayish-blue
	Morphine SO_4	Purplish-gray
	Morphine HCl	Purplish-gray
	Morphine PO_4	Purplish-gray
	Meperidine HCl	Colorless
	Phenazocine HBr	Reddish-violet
p-Dimethylamino-benzaldehyde, (10% in glacial acetic acid)	Codeine SO_4	Orange turning to pink
	Codeine HCl	Orange becoming pink
	Codeine PO_4	Orange turning to pink
	Morphine SO_4	Orange becoming pink
	Morphine HCl	Orange becoming pink
	Morphine PO_4	Orange turning to pink
	Meperidine HCl	Colorless
	Phenazocine HBr	Violet changing to reddish-violet

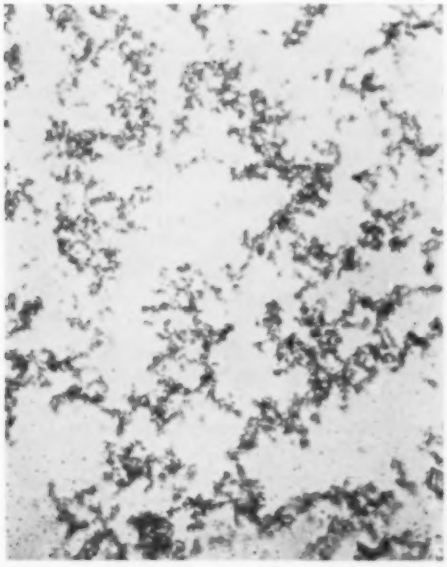


Fig. 2.—Phenazocine hydrobromide 1:1000 with mercuric-potassium iodide T. S.

(crystalline potassium) will also react with phenazocine to form a precipitate, a reaction that does not occur with codeine salts, morphine salts, or meperidine hydrochloride.

Microchemical.—Phenazocine hydrobromide, codeine sulfate, morphine sulfate, and meperidine hydrochloride may be identified microchemically through formation of characteristic crystals with mercuric-potassium iodide T. S. (U. S. P.) using the Clarke-Williams microtechnique (8).

TABLE III.—COLOR REACTIONS WHICH DISTINGUISH AQUEOUS SOLUTIONS OF PHENAZOCINE HYDROBROMIDE FROM AQUEOUS SOLUTIONS OF CODEINE SALTS, MEPERIDINE HYDROCHLORIDE, AND MORPHINE SALTS

Reagent or T.S.	Aqueous Drug Solution	Color Reaction
Selenous acid (0.5% in H_2O)	Codeine SO_4, 30 mg./ml.	Bluish-green turning to dark green
	Codeine HCl, 30 mg./ml.	Bluish-green turning to dark green
	Codeine PO_4, 30 mg./ml.	Bluish-green changing to dark green
	Morphine SO_4, 5 mg./ml.	Olive-green turning to grayish-green
	Morphine HCl, 5 mg./ml.	Olive-green turning to grayish-green
	Morphine PO_4, 5 mg./ml.	Olive-green changing to grayish-green
	Meperidine HCl, 75 mg./ml.	Colorless
	Phenazocine HBr, 2 mg./ml.	Light gray
Sodium tungstate (1% in H_2O)	Codeine SO_4, 30 mg./ml.	Dark bluish-purple
	Codeine HCl, 30 mg./ml.	Purple or grayish-purple
	Codeine PO_4, 30 mg./ml.	Dark bluish-purple
	Morphine SO_4, 5 mg./ml.	Grayish-purple
	Morphine HCl, 5 mg./ml.	Grayish-purple
	Morphine PO_4, 5 mg./ml.	Grayish-purple
	Meperidine HCl, 75 mg./ml.	Colorless
	Phenazocine HBr,2 mg./ml.	Reddish-violet changing to grayish-red
p-Dimethyl-aminobenzalde-hyde (10% in glacial acetic acid)	Codeine SO_4, 30 mg./ml.	Orange turning deep red
	Codeine HCl, 30 mg./ml.	Orange turning deep red
	Codeine PO_4, 30 mg./ml.	Orange changing to deep red
	Morphine SO_4, 5 mg./ml.	Orange-red
	Morphine HCl, 5 mg./ml.	Orange-red
	Morphine PO_4, 5 mg./ml.	Orange-red
	Meperidine HCl, 75 mg./ml.	Light tan
	Phenazocine HBr, 2mg./ml.	Light violet turning reddish-violet

Procedure.—Prepare 0.1% solutions of each of the drugs in 1% aqueous hydrochloric acid. Place a microdrop (approx. 0.1 cu. mm.) of the sample on a cover slip, add a similar drop of mercuric-potassium iodide T. S. (U. S. P.), and mix. Invert cover slip and place on a cavity slide. Examine the hanging drop under a microscope. As soon as a precipitate is noticed, ring the cover slip with 25% acacia solution to prevent further evaporation and allow samples to stand overnight.

The characteristic crystals formed for each of the drugs are shown in Figs. 2–5.

The hydrochloride and phosphate salts of codeine and morphine form similar crystals to those of codeine sulfate and morphine sulfate, respectively. Additional crystal tests which will distinguish phenazocine from its (-) isomer have been published by Clarke (7).

TABLE IV.—PRECIPITATION REACTIONS WHICH DISTINGUISH AQUEOUS SOLUTIONS OF PHENAZOCINE HYDROBROMIDE FROM AQUEOUS SOLUTIONS OF CODEINE SALTS, MORPHINE SALTS, AND MEPERIDINE HYDROCHLORIDE

	Test Solution		
Drug, 0.5% Aqueous Solution	Palladium Chloride, 1% in 15% HCl	Sodium Nitroprusside, 5% in H_2O	Penicillin G, Cryst. Potass., 10% in H_2O
Phenazocine hydrobromide	Brownish, amorphous precipitate	White, amorphous precipitate	White, amorphous precipitate
Codeine sulfate	No reaction	No reaction	No reaction
Codeine hydrochloride	No reaction	No reaction	No reaction
Codeine phosphate	No reaction	No reaction	No reaction
Morphine sulfate	No reaction	No reaction	No reaction
Morphine hydrochloride	No reaction	No reaction	No reaction
Morphine phosphate	No reaction	No reaction	No reaction
Meperidine hydrochloride	No reaction	No reaction	No reaction

Fig. 3.—Codeine sulfate 1:1000 with mercuric-potassium iodide T. S.

Dosage Forms

Because phenazocine hydrobromide is only slightly, and very slowly, soluble in water, propylene glycol has been included in the ampul and multiple dose vial formulas to aid in solubilization. Simple aqueous solutions of phenazocine hydrobromide are partially decomposed by autoclaving. However, if the solutions are buffered at a pH of approximately 3, autoclaving can be conducted without chemical destruction. A sodium citrate-citric acid buffer is

Fig. 4.—Morphine sulfate 1:1000 with mercuric-potassium iodide T. S.

utilized for this purpose. The ampul formula is adjusted to isotonicity with sodium chloride, whereas the multiple dose vial formula is isotonic without the addition of this ingredient. The multiple dose vial formula contains benzyl alcohol as a preservative. The formulas are as follows:

Ampul

Ingredients	% w/v
Phenazocine hydrobromide	0.25[a]
Sodium citrate	0.13
Citric acid	0.37
Sodium chloride	0.10
Propylene glycol	1.50 v/v
Water for injection *q. s. ad*	100.00

Multiple Dose Vial

Ingredients	% w/v
Phenazocine hydrobromide	0.25[a]
Sodium citrate	0.13
Citric acid	0.37
Propylene glycol	1.00 v/v
Benzyl alcohol	1.00 v/v
Water for injection *q. s. ad*	100.00

[a] Equivalent to 2 mg. free base per ml.

Stability

The stability of phenazocine hydrobromide in the terminally autoclaved ampul and multiple-dose vial formulations was evaluated over prolonged intervals at varying temperatures. No objectionable color changes, precipitation, or pH changes occurred after storage for one month at −15° and direct sunlight, three months at 45°, six months at 5° and 37°, and one year at RT. The chemical stability of the drug in these formulations has been summarized in Table V. Table VI contains stability data on phenazocine hydrobromide stored in dry powder form at varying temperatures.

TABLE V.—STABILITY OF PHENAZOCINE IN AMPUL AND MULTIPLE-DOSE VIAL FORMULATIONS

Formulation	Storage Temp., °C.	Storage Period	Assay, % Label Claim
Ampul, 2 mg./ml.	Initial		100
	60	14 days	99
	45	2 mo.	100
	37	6 mo.	99
	RT	1 yr.	101
	−15	1 mo.	100
Multiple-dose vial, 2 mg./ml.	Initial		100
	60	14 days	100
	45	3 mo.	100
	37	6 mo.	101
	RT	1 yr.	100
	−15	1 mo.	99

TABLE VI.—STABILITY OF PHENAZOCINE HYDROBROMIDE

Storage Temp., °C.	Storage Period	Assay, % of Purity
Initial		101
60	1 mo.	98
45	3 mo.	100
37	6 mo.	99
RT	12 mo.	101

Physical Compatibility of Parenteral Forms of Phenazocine Hydrobromide

The commercially available ampul and multiple-dose vial formulations of phenazocine hydrobromide are in most cases physically compatible with other commercial injectables which are acidic or neutral in nature. Strongly alkaline products, such as parenteral barbiturate preparations, will cause precipitation of the water-insoluble base. Penicillin preparations will also react with phenazocine hydrobromide to form an insoluble precipitate.

SUMMARY

1. Formulations were developed for the preparation of ampul and multiple-dose vial solutions of phenazocine hydrobromide.

2. The phenazocine hydrobromide in these dosage forms has been found to be very stable in both accelerated and long term stability studies.

Fig. 5.—Meperidine hydrochloride 1:1000 with mercuric-potassium iodide T. S.

3. Color tests, precipitation reactions, and microchemical crystal formations, which may be used to distinguish phenazocine hydrobromide from codeine salts, meperidine hydrochloride, and morphine salts, were developed.

4. X-ray diffraction data and infrared curves on three crystalline forms of phenazocine hydrobromide are included.

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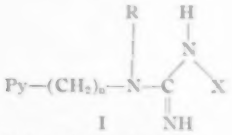
Pyridylalkyl Dicyandiamides, Biguanides, and Guanylureas

By SEYMOUR L. SHAPIRO, ELAINE S. ISAACS, and LOUIS FREEDMAN

A series of pyridylalkyl dicyandiamides, guanylureas, and biguanides has been synthesized and the compounds evaluated for anti-inflammatory activity and potentiation of the hexobarbital effect.

PYRIDYLALKYL or pyridyl derivatives of polynitrogen systems have been examined as hypoglycemic agents (biguanides) (1), anti-inflammatory agents (aminoguanidines)(2), analgesics and anticonvulsants (guanylpiperazines) (3), and antimalarials (guanidines) (4).

New variants (I) of such structures have now been synthesized and explored for pharmacological activity



Py = 2, 3, and 4-pyridyl
n = 0–2
R = H and CH_3
X = —CN (dicyandiamides)
—$CONH_2$ (guanylureas)
—$C(NH)NHC_6H_4$-pCl (biguanides)

The pyridylalkyl dicyandiamides (I, X = CN) were conveniently obtained by reacting the picolyl or pyridylethylamine hydrochloride with sodium dicyanamide in refluxing butanol (5). These compounds in turn were converted to the guanylureas (I, X = $CONH_2$) by hydrolysis with hydrochloric acid in isopropyl alcohol (6). Upon reaction with the aniline hydrochloride in water, the pyridylalkyl dicyandiamide afforded the N^1-pyridylalkyl-N^5-arylbiguanide (7) which was obtained as mixtures of mono- and di-hydrochlorides and which could not be purified and were characterized as their dipicrates.

The pyridyl nitrogen in these biguanides provides a site for addition of a third proton (6), but apparently pKa values are not sufficiently discrete for isolation of well defined mono or polyhydrochlorides. Alternatively, while N^1,N^5-biguanides give monopicrates (8), here the isolation of dipicrates suggests that the most basic site is in the biguanide nucleus, followed by the pyridine nitrogen, with the lowest order of basicity being a second basic site in the biguanide nucleus.

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The compounds synthesized are shown in Table I.

The pyridylalkyl dicyandiamides are relatively nontoxic compounds ($LD_{min.}$ 750–1,000 mg./Kg.) and show potentiation of hexobarbital time (9), and anti-inflammatory effects (10). Of interest is the comparatively high toxicity ($LD_{min.}$ 20 mg./Kg.) of the 4-pyridyl dicyandiamide. The N-methyl-dimethylaminopropyl dicyandiamide (compound 11) was nontoxic but was without pharmacological effect in a variety of tests.

The pyridylalkylguanylureas ($LD_{min.}$ 250–750 mg./Kg.) afforded significant hypotension (11) with compounds 12 and 13, whereas compounds 17 and 18 were hypertensive and potentiators of epinephrine (12). Compound 14 showed adrenergic block. The N^1-pyridylalkyl-N^5-p-chlorophenyl biguanides showed good anti-inflammatory activity, particularly compounds 22 and 23 (evaluated as the hydrochloride salts).

EXPERIMENTAL[1]

General Procedure for Preparation of the Pyridylalkyl Dicyandiamides.—A mixture of 0.5 mole of the pyridylalkyl amine in 500 ml. of n-butanol, 42 ml. (0.5 mole) of hydrochloric acid, and 55.0 Gm. (0.5 mole) of sodium dicyanamide was stirred under reflux over six hours. The formed sodium chloride was separated from the hot solution and after removal of the butanol, the residue of product was recrystallized. The yields ranged from 31 to 67%.

General Procedure for the Preparation of the Picolyl and Pyridylethyl Guanylureas.—A suspension of 0.025 mole of the pyridylalkyl dicyandiamide in a mixture of 12 ml. of isopropyl alcohol and 6 ml. of 12 N hydrochloric acid was heated under reflux, with stirring, over a period of two to three hours. When cool, the formed product, as the dihydrochloride, separated and was filtered and recrystallized. When the product did not so crystallize, the reaction mixture was diluted with water (70 ml.) and neutralized (methyl red) with 10 N sodium hydroxide and 5.3 Gm. of sodium nitrate added. The precipitated nitrate was separated and recrystallized. Yields ranged from 18 to 39%.

N^1-Methyl-N^1-(4-pyridylethyl)-N^5-(p-chlorophenyl)-biguanide.—A mixture of 1.9 Gm. (0.015 mole) of p-chloroaniline in 4.9 ml. (0.03 mole) of 6 N hydrochloric acid and 3.0 Gm. (0.015 mole) of N-methyl-(4-pyridylethyl)-dicyandiamide was heated under reflux for five hours. When cool, the product precipitated as a glassy solid and was

[1] The pyridylalkyl amines were obtained from Sapon Laboratories, Lynbrook, N. Y.

TABLE I.—PYRIDYLALKYL DERIVATIVES (I)

No.	Py[a]	n	R	HX	M. P. °C.[b]	RS[c]	Formula	Analysis, %[d] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
							X = —CN						
1	2	1	H	...	142–145	A	$C_8H_9N_3$	54.8	54.1	5.2	5.1	...	...
2	3	1	H	...	147–150	B	$C_8H_9N_3$	54.8	54.6	5.2	5.4	40.0	39.8
3[e]	4	1	H	...	78–81	A	$C_8H_{11}N_3O$	49.7	49.9	5.7	5.7	...	...
4	2	1	CH_3	...	150–152	B	$C_9H_{11}N_3$	57.1	57.1	5.9	5.7	37.0	37.1
5	3	1	CH_3	...	158–160	B	$C_9H_{11}N_3$	57.1	57.2	5.9	6.0	37.0	37.1
6	2	2	H	...	143–146	B	$C_9H_{11}N_3$	57.1	57.2	5.9	6.0	37.0	37.3
7	4	2	H	...	194–195	B	$C_9H_{11}N_3$	57.1	57.2	5.9	6.0	37.0	37.3
8	2	2	CH_3	...	165–168	A	$C_{10}H_{12}N_3$	59.1	58.9	6.5	6.7	...	...
9	4	2	CH_3	...	202–204	A	$C_{10}H_{12}N_3$	59.1	58.8	6.5	6.3	...	...
10	4	0	H	...	114–115	C	$C_7H_7N_3$	...	...	...	...	43.6	44.3
11[f]	..	..	CH_3	...	119–121	B	$C_8H_{17}N_3$	52.4	52.3	9.4	9.4	38.2	38.4
							X = —$CONH_2$						
12	2	1	H	2HCl	211	D	...	...	...	...	...	...	...
13	2	1	H	2HPic	193–194	E	$C_{14}H_{14}N_6O_9$	36.9	36.8	2.6	2.6	23.7	24.1
14	3	1	H	HNO_3	175–177	E	$C_8H_{12}N_6O_4$	37.5	37.7	4.7	4.3	32.8	33.0
15	2	2	H	2HCl	198–200	F	$C_9H_{13}Cl_2N_3O$	38.6	39.2	5.4	5.4	25.0	24.7
16	4	2	H	2HCl	220–221	G	$C_9H_{13}Cl_2N_3O$	38.6	38.5	5.4	5.2	25.0	24.6
17	2	2	CH_3	2HCl	183–184	H	$C_{10}H_{17}Cl_2N_3O$	40.8	40.6	5.8	6.0	23.8	23.6
18	4	2	CH_3	HNO_3	189–191	B	$C_{10}H_{16}N_6O_4$	42.3	43.1	5.7	5.7	29.6	29.5
							X = C(NH)NHC_6H_4-*p*Cl						
19	2	1	H	2HPic	200–202	E	$C_{26}H_{23}ClN_{12}O_{14}$	41.0	41.4	2.8	2.6	22.1	22.3
20	2	1	CH_3	2HPic	194–196	E	$C_{27}H_{25}ClN_{12}O_{14}$	41.8	41.9	3.0	3.8	...	...
21	3	1	CH_3	2HPic	207–210	E	$C_{27}H_{25}ClN_{12}O_{14}$	41.8	42.1	3.0	3.2	...	...
22	2	2	H	2HPic	182–184	B	$C_{27}H_{25}ClN_{12}O_{14}$	41.8	41.3	3.0	3.0	...	...
23	2	2	CH_3	2HPic	211–212	E	$C_{28}H_{27}ClN_{12}O_{14}$	42.6	43.3	3.2	3.1	...	...
24	4	2	CH_3	2HPic	209–211	E	$C_{28}H_{25}ClN_{12}O_{14}$	...	...	...	...	21.3	21.3

[a] The number indicates position of pyridyl nitrogen. [b] Melting points are not corrected and were determined on a Fisher-Johns melting point block. [c] RS is recrystallizing solvent: A, acetonitrile; B, isopropanol-hexane; C, ethanol-ether; D, methanol-water; E, water; F, methyl cellosolve-acetonitrile; G, methyl cellosolve; H, methanol-benzene. [d] Analyses are by Weiler and Strauss, Oxford, England. [e] Isolated, and analyses shown as monohydrate. [f] The pyridylalkyl group is replaced by $(CH_3)_2N(CH_2)_2$—.

separated, and after solution in hot water, on cooling, gave 2.7 Gm. of product, m. p. 212–213°. Analyses indicated a mixture of mono and dihydrochlorides.

The compound was characterized as its dipicrate (compound 24).

The other biguanides were processed and characterized in a similar manner.

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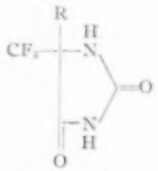
5-Trifluoromethyl Hydantoins

By BERNARD LOEV and MINERVA FLORES

NUMEROUS COMPOUNDS have been made in which the trifluoromethyl group has been placed on the aromatic ring of biologically active agents, in order to examine the effect of this group on biological activity (1). However, there are few instances in which the effect of this incorporation into aliphatic compounds has been studied. We wish to report here the synthesis of two trifluoromethyl substituted hydantoins, prepared for comparison with the methyl analogs.

The trifluoromethyl ketone or cyanohydrin, on heating with ammonium carbonate and potassium cyanide in a sealed container, gave the 5-trifluoromethylhydantoins. When the same mixture was heated at atmospheric pressure, no reaction occurred.



I, R = CH_3
II, R = C_6H_5

The trifluoromethyl compounds retained the biological activity of their methyl analogs (2).[1] Oral administration of compounds I and II to mice produce CNS depression characterized by reduction in motor activity, hypotonia, ataxia, and dyspnea, at doses starting with 1,000 and 200 mg./Kg., respectively. At higher doses, marked hypothermia was observed. Compound II produced death within three hours at 1,000 mg./Kg. I did not block the tonic extensor phase of the maximal electroshock seizure pattern in mice. II has an ED_{50} of 35 mg./Kg., p. o., in the same test; this is essentially the same as the corresponding methyl compound.

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[1] 5,5-Dimethylhydantoin was purchased from Aldrich Chemical Co.

EXPERIMENTAL

5 - Methyl - 5 - trifluoromethylhydantoin (I).—A mixture of 21 Gm. (0.15 mole) trifluoroacetone cyanohydrin, 43 Gm. (0.45 mole) ammonium carbonate, and 1.55 Gm. (0.0225 mole) potassium cyanide in 50 ml. of 50% aqueous ethanol was heated in a closed vessel at 105° for two days. The volatile materials were removed by heating *in vacuo* at 100°, then the residue was taken up in a little water, neutralized with dilute hydrochloric acid, and concentrated again *in vacuo*. The solid residue was extracted several times with hot alcohol, and the extracts were concentrated at reduced pressure to give an oil which was chromatographed over alumina using tetrahydrofuran as solvent. The product was a solid, 5.4 Gm. (20% yield), m. p. 136–138°.

Anal.—Calcd. for: $C_5H_5F_3N_2O_2$: C, 32.98; H, 2.77; N, 15.38. Found: C, 33.46; H, 3.28; N, 15.54.

5 - Phenyl - 5 - trifluoromethylhydantoin (II).—A suspension of trifluoroacetophenone (13 Gm., 0.075 mole), potassium cyanide (7.75 Gm., 0.1125 mole), and ammonium carbonate (216 Gm., 0.225 mole) in 50% aqueous ethanol was heated in a sealed vessel at 95° for twenty-four hours. The volatile materials were removed by heating *in vacuo* at 100°, and the residual solid was recrystallized from aqueous methanol, m. p. 231–235°, 6.6 Gm., 36% yield. The compound showed a single spot on paper chromatography and a nonaqueous titration showing 99.9% purity.

Anal.—Calcd. for: $C_{10}H_7F_3N_2O_2$: C, 52.17; H, 3.0. Found: C, 52.0; H, 2.9.

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Effect of Potassium, Nitrogen, and Phosphorus on the Growth and Glycoside Accumulation in *Digitalis lanata* I. Leaf Yield and Total Glycosides

By D. P. N. TSAO, L. M. GOMEZ, and M. G. CHAUBAL

Plants of *Digitalis lanata* were grown in experimental soil cultures with a high level of potassium, nitrogen, and phosphorus. It was observed that plants grown on the high level phosphorus produced a significantly less growth. No significant effects in total glycoside accumulation were observed in plants grown in high level potassium and nitrogen. On the other hand, leaf material from plants grown with a high level phosphorus showed a significant decrease in total glycoside yield.

THE INFLUENCE of fertilizers upon the growth of *Digitalis* species has been investigated by a number of workers and a number of controversial results have been reported (1–4). Courth and Allemann (1) indicated a decrease in activity in leaves of *Digitalis lanata* Ehrh. when plants were treated with stable manure, whereas artificial fertilizers, especially those containing nitrogen, produced an increase in glycoside activity. They also stated that these plants grew naturally in Switzerland on lime-containing dolomitic soil but when they were cultivated on light, humus-containing soil, poor in lime, the crop yield and glycoside activity were much higher. Boshart (1) reported that ammonium sulfate was very useful for the growth of *Digitalis purpurea*, but Dauphinee (2) did not find it of significant value. Mascre and Lefrancois (3) considered potassium, nitrogen, and phosphorus to have a favorable effect upon the leaf yield of *D. purpurea*. On the other hand, Tsao and Youngken (4) showed that potassium chloride had no significant effect on the growth and glycoside accumulation of this plant in hydroponic culture. Rowson (1) found that phosphatic fertilizer alone and in association with potassium sulfate or ammonium sulfate might produce a small increase in leaf yield and in glycosidal content in the leaves of *D. purpurea*.

Such a conflict of findings upon the influence of artificial fertilizer and inorganic ions upon leaf yield and a limited number of results dealing with their glycoside yield have led to the present investigation.

EXPERIMENTAL

Seeds of *Digitalis lanata* Ehrh. were germinated in the greenhouse in a culture flat containing vermiculite on February 23, 1960. A modified Hoagland's solution was fed as nutrient (5). Sixty-eight days after germination, 48 plants of approximately the same size and leaf development were transplanted into the experimental plot in the field of the drug plant garden of the University of Rhode Island. The experimental plot was divided into four beds (20 sq. ft. for each bed). Each bed had 12 plants. Bed No. 1 was used as control. Beds No. 2, 3, and 4 were assigned for fertilizing with a high level of potassium, nitrogen, and phosphorus. Each bed received 125 Gm. of complete 7-7-7 fertilizer. Three experimental beds (No. 2–4) received a treatment of potassium chloride, 20 Gm.; ammonium nitrate, 40 Gm.; and superphosphate (20% available phosphorus pentoxide), 63 Gm. per 20 sq. ft., respectively. Top dressing of high level potassium, nitrogen, and phosphorus was applied on experimental beds on May 25 and 31, June 16, and August 17, 1960. The observation of the growth of *Digitalis lanata* plants were recorded (See Figs. 1 and 2). On August 12, 1960, the plants in bed No. 4 (excess phosphorus) were smaller though normal and healthy appearing. One plant in this bed showed a flowering stalk (see Fig. 1). The plants from these four beds were harvested on September 22, 1960. The weight of fresh leaves from each plant was recorded. The fresh materials were then transferred immediately into a hot air circulating dryer and dried at a temperature of 35–40°. Dry weight and moisture content were measured. The dried leaves were ground into No. 60 powder and stored in an air-tight container for gylcoside analyses.

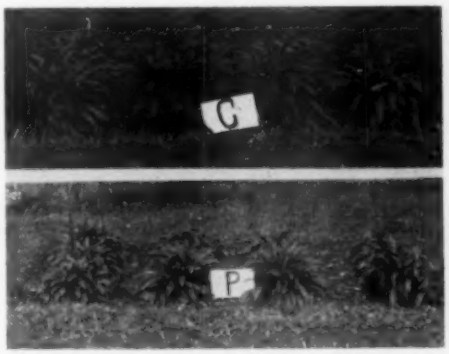

Fig. 1—Growth effect produced by high level phosphorus treatment in *Digitalis lanata*.

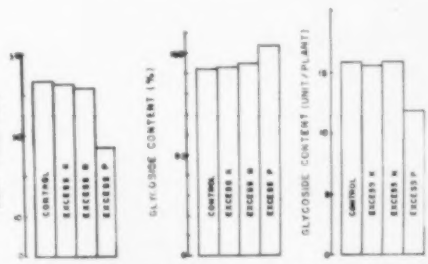

Fig. 2—Leaf yield and glycoside content of *Digitalis lanata* feeding with high level potassium, nitrogen, and phosphorus.

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TABLE 1—STATISTICAL ANALYSES OF LEAF YIELD AND GLYCOSIDE FORMATION FOLLOWING CHEMICAL FEEDINGS OF *Digitalis lanata* Ehrh.

Group	No. of Plants	Leaf Yield: Dry Wt., Gm.	Leaf Yield: Difference, %	Total Glycoside Content: Potency,[a] %	Total Glycoside Content: Difference, %	Total Glycoside Content: *t* Test	Total Glycoside Content: Potency,[b] unit/plant	Total Glycoside Content: Difference, %	Total Glycoside Content: *t* Test
Control	12	43.1 ± 3.0	...	91.7 ± 2.8	...	...	15.7 ± 1.1	...	...
Excess potassium	12	42.4 ± 3.8	−2	92.1 ± 2.3	0	0.1[c]	15.5 ± 1.4	−1	0.1[c]
Excesss nitrogen	12	41.5 ± 3.1	−4	94.1 ± 2.7	3	0.6[c]	15.7 ± 1.4	0	0.2[c]
Excess phosphorus	12	27.3 ± 2.0	−37	102.6 ± 2.6	12	3[d]	11.6 ± 0.9	−26	3[d]

[a] In comparison with a reference standard of *Digitalis lanata* leaves (expressed as 100%). [b] Unit = leaf yield × 4 × potency in %/100. [c] Not significant at 1% level. [d] Significant at 1% level.

All *Digitalis lanata* tinctures were prepared according to the U. S. P. XVI method (6) with the modification of the ratio of dried leaves to menstruum (alcohol-water 4:1) 1:20 instead of 1:10. The total glycoside was determined by using a modified Knudson-Dresbach method (7). The absorbance was read at 525 mμ in a standard cell by the use of a Bausch and Lomb Spectronic-20. A standard curve was constructed from a tincture prepared from *Digitalis lanata* leaves.[1] Unknown tinctures were then compared with this tincture and the glycoside yields were expressed in per cent potency of the latter standard, expressed as 100%.

The dried leaf yield and glycoside content (expressed in per cent) of four groups (control, excess potassium, nitrogen, and phosphorus) are shown in Table I and Fig. 2. The glycoside content was also calculated in units for each individual plant. The unit was derived from the following formula

$$\text{unit per plant} = \text{dried leaf yield} \times 4 \times \frac{\text{glycoside content in per cent}}{100}$$

A *t* test is applied to glycoside yields as shown in Table I.

DISCUSSION

The data (Table I and Fig. 2) revealed that *Digitalis lanata* grown in high level phosphorus showed a decrease in total weight of leaves by 37% in comparison with the control; plants treated with high level potassium and nitrogen indicated no significant difference in comparison with the controls. The leaf material from high level potassium and nitrogen fertilizing showed no difference in total glycoside yield in comparison with the controls. Plants treated with a high level of phosphorus showed an increase in glycoside accumulation by 12%. As the results showed the glycoside content calculated as units per plant for the same group was 26% lower than the control. If phosphorus actually stimulated glycoside synthesis, the figures for glycoside content (see Table 1) (units per plant) of excess phosphorus group should be >16 instead of <16 units. This study indicated that high level of phosphorus inhibited growth and glycoside formation in *Digitalis lanata*.

[1] The authors are indebted to Burroughs Wellcome & Co., Tuckahoe, N. Y., for the supply of *Digitalis lanata* material.

SUMMARY

The effect of potassium, nitrogen, and phosphorus on the growth and glycoside synthesis in *Digitalis lanata* has been studied.

1. Plants treated with high level potassium and nitrogen levels showed no significant difference in leaf yield and glycoside formation in comparison with controls.

2. High level phosphorus inhibited the leaf yield and glycoside synthesis significantly as shown by the *t* test applied to the data.

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Tableting of a Chemical with Variable Color Characteristics

By S. J. TUCKER and F. A. OYER

A method is presented which permits the tableting of chemicals which vary slightly in color from lot to lot. The procedure involves the suspending of the active ingredient, and diluents if needed, in a methylcellulose solution containing a dye. The material is then spray dried, mixed with disintegrating agents and lubricant, and compressed.

OCCASIONALLY, the development pharmacist is confronted with the problem of having to prepare a tablet dosage form using a raw material which varies slightly in color from lot to lot. The impurity causing the variation may be biologically insignificant and of such a nature as to be undetectable analytically and impractical to remove chemically. If the dose of the active ingredient is small, the problem is minimized since the diluents will usually overcome the difficulty. However, if the dose is large, dilution is no longer effective.

In general, there are two methods presently used to solve this problem. (*a*) Inclusion of an added colorant in the granules. This is helpful but not always effective, since during granulating it becomes a matter of chance whether the color will cover the drug or the drug will cover the color. (*b*) Coating of the finished tablet. This is effective but additional expense is required.

It has been found that by employing the technique of granulation by spray drying moderate color variation from lot to lot can be overcome.

DISCUSSION

The most effective way to mask a color is either to coat the tablet as a whole unit or to coat the individual particles. Coating of the individual particle and granulating can be accomplished at the same time by use of spray drying. The spray drying of granulations has been described by Robinson, *et al.* (1). The economics of spray drying are well known in the soap and soluble coffee industries. Although a recent article (2) indicates that spray drying is impractical, this laboratory has found that spray drying has great potentiality and usefulness in many varied applications.

Essentially, the technique consists of suspending the active drug and diluents in a solution containing methylcellulose and a dye (the color and amount depending upon the objective). The suspension is then spray dried, the methylcellulose and the dye thus coat the individual particles.

PROCEDURE AND RESULTS

Three hundred grams of a therapeutic agent and 150 Gm. of terra alba were suspended in a solution containing 1.2 Gm. of FD&C yellow No. 5, 450 ml. of a 3% Methocel (15 cps.) solution, 75 ml. of SD No. 30 alcohol, and 350 ml. of water. The material was then spray dried using a Niro Model Minor spray dryer under the following conditions: inlet temperature, 165°; outlet temperature, 72°; atomizer pressure, 6.2 Kg./cm.^{2}.

This procedure was repeated using a different batch of the same therapeutic agent. The two batches of chemicals were visibly different in color shades.

The two finished powders were no longer visibly distinguishable, and the similarity was maintained after compression.

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Gas Chromatography of Barbiturates and Related Compounds

Sir:

Methods for separating and identifying micro quantities of barbiturates and related compounds are of great importance for work in pharmacy, pharmacology, and toxicology. Paper chromatographic methods, which are most extensively used at the present time, are slow and do not possess the high sensitivity that is often desirable. Furthermore, there are several combinations which are not adequately resolved by this technique.

Attempts to identify the barbiturates by gas chromatography have been reported by Janák (1, 2). He pyrolyzed their sodium salts and gas chromatographed the pyrolysis products.

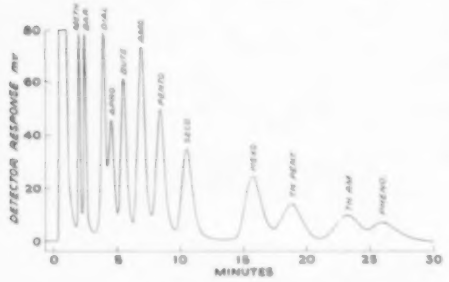

Fig. 1.—Gas chromatogram of twelve barbiturates on Apiezon L. Temp., 150°; column, 6 feet long, 3 mm. i.d.; pressure, 20 p.s.i.; flow rate, 37.5 ml./min. METH., metharbital; BARB., barbital; DIAL., diallyl barbituric acid; APRO., aprobarbital; BUTE., butethal; AMO., amobarbital; PENTO., pentobarbital; SECO., secobarbital; HEXO., hexobarbital; TH. PENT., thiopental; TH. AM., thiamylal; PHENO., phenobarbital.

Direct gas chromatographic separation of barbiturates has now been carried out on a number of stationary phases, including silicone rubber, Dow Corning fluorosilicone fluid, Apiezon, polyesters, and polyethylene glycol.

The stationary phases are coated in 1 to 2 per cent concentration on acid- and alkali-washed Chromosorb W. An argon β-ionization detector has been used in this work. A typical sample is 1.0 μL. containing 0.5 to 1.0 per cent of each barbiturate. The gas chromatographic behavior of twenty-one barbiturates and two related compounds, glutethimide and allylisopropylacetylurea, has been investigated. The substances give single component sharp peaks consistent with absence of decomposition. A chromatogram showing the power of this method is illustrated in Fig. 1. Pentobarbital and amobarbital, which are very difficult to separate by paper chromatography, are easily separated on this column containing Apiezon L as the stationary phase. Because of its great sensitivity and resolving power, it is suggested that this method will be useful for work with biological systems.

A complete report will be published shortly.

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E. Brochmann-Hanssen
A. Baerheim Svendsen

University of California
School of Pharmacy
San Francisco, Calif.

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Contents continued from back cover

SISTER M. LAWRENCE O'DONNELL, ANNA MAE SEBULAK, CORNELIUS W. KREKE — 764 — Estimation of Mercurial-Protein Binding by a Polarographic Method

S. L. SHAPIRO, H. SOLOWAY, H. J. SHAPIRO, L. FREEDMAN — 769 — Local Anesthetics III. Dialkylaminoalkoxyphenylethanol Esters

A. M. BURKMAN — 771 — Relative Potencies of Some Phenothiazines as Pecking Syndrome Inhibitors

F. M. GOYAN, R. D. JOHNSON, R. H. BLACKWOOD — 773 — Incremental Thermal Titrations

C. E. BRECKINRIDGE, JR., J. E. CHRISTIAN — 777 — Study of Inverse Isotope Dilution Analyses of Chlortetracycline

MARILDA M. DE OLIVEIRA, SYLVIA O. ANDRADE — 780 — Toxic Saponin from *Elvira biflora*

HAROLD ZALLEN, JOHN E. CHRISTIAN, ADELBERT M. KNEVEL — 783 — Polarographic Determination of the Rates of Formation of Ethyleneimonium Ions of Several Nitrogen Mustard Compounds

Technical Articles

DUNCAN E. MCVEAN, ALBERT M. MATTOCKS — 785 — Simple Rheometer for Definition of Viscoelastic Properties of Structural Liquids

NORMAN L. HENDERSON, PAUL M. MEER, H. B. KOSTENBAUDER — 788 — Approximate Rates of Shear Encountered in Some Pharmaceutical Processes

Drug Standards

W. E. LATSHAW, D. R. MACDONNELL — 792 — Phenazocine Hydrobromide: Pharmaceutical Aspects of a New Benzomorphan Analgesic Related to Morphine

Notes

SEYMOUR L. SHAPIRO, ELAINE S. ISAACS, LOUIS FREEDMAN — 798 — Pyridylalkyl Dicyandiamides, Biguanides, and Guanylureas

BERNARD LOEV, MINERVA FLORES — 800 — 5-Trifluoromethyl Hydantoins

D. P. N. TSAO, L. M. GOMEZ, M. G. CHAUBAL — 801 — Effect of Potassium, Nitrogen, and Phosphorus on the Growth and Glycoside Accumulation in *Digitalis lanata* I. Leaf Yield and Total Glycosides

S. J. TUCKER, F. A. OYER — 803 — Tableting of a Chemical with Variable Color Characteristics

Communications

E. BROCHMANN-HANSSEN, A. BAERHEIM SVENDSEN — 804 — Gas Chromatography of Barbiturates and Related Compounds

JOURNAL OF **Pharmaceutical Sciences**

September 1961 volume 50, number 9

Editorial

I What's in a Name?

Review Article

JACK LAZARUS, JACK COOPER — 715 Absorption, Testing, and Clinical Evaluation of Oral Prolonged-Action Drugs

Research Articles

ALEXANDER H. C. CHUN, ALFRED N. MARTIN — 732 Measurement of Hydrophile-Lipophile Balance of Surface-Active Agents

JORDAN G. LEE, JOSEPH A. LIUZZO, ARTHUR F. NOVAK — 737 Alfalfa Extracts Which Stimulate Growth of *Neurospora sitophila* I. Preparation and Activation of Crude Extracts

JORDAN G. LEE, JOSEPH A. LIUZZO, ARTHUR F. NOVAK — 739 Alfalfa Extracts Which Stimulate Growth of *Neurospora sitophila* II. Isolation, Characterization, and Multiple Nature of Stimulatory Compounds

JORDAN G. LEE, ARTHUR F. NOVAK, JOSEPH A. LIUZZO — 74 Alfalfa Extracts Which Stimulate Growth of *Neurospora sitophila* III. Effect on Weight Gain of Thyroid-Stressed Albino Rats

DURWARD N. ENTREKIN — 743 Relation of pH to Preservative Effectiveness I. Acid Media

HARALD G. O. HOLCK, MARVIN H. MALONE, HIDEKO KATAYAMA KAJI — 747 Pharmacologic Study of Alkyl 2-Bromoallyl Barbituric Acids

BERNARD A. HAINES, JR., ALFRED N. MARTIN — 753 Interfacial Properties of Powdered Material; Caking in Liquid Dispersions II. Electrokinetic Phenomena

BERNARD A. HAINES, JR., ALFRED N. MARTIN — 756 Interfacial Properties of Powdered Material; Caking in Liquid Dispersions III. Adsorption Studies

DON S. BENSLAY, ROB S. MCCUTCHEON — 760 Some Cardiovascular Effects of Trimethobenzamide

Contents continued on inside back cover